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(71)(72) Applicants and Inventors: HOUEN, Gunnar [DK/DK]; Wienerbyen 14, DK-2830 Virum (DK). HOLM, Arne [DK/DK]; Margrethevej 19, DK-2840 Holte (DK).

(74) Agent: HOFMAN-BANG & BOUTARD A/S; Adelgade 15, DK-1304 Copenhagen K (DK).

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(54) Title: A METHOD FOR THE STEPWISE, CONTROLLED SYNTHESIS OF CHEMICAL SPECIES, PARTICULARLY PEPTIDES, COUPLED PRODUCTS OBTAINED BY THE METHOD AND THE USE OF THESE COUPLED PRODUCTS, E.G. AS VACCINES

(57) Abstract

Chemical species, particularly peptides, are synthesized by a stepwise, controlled process, in which a proteinaceous substance such as a protein is used as the synthesis substrate. The products obtained by the process can be used e.g. as vaccines against various diseases and as matrix materials or carrier molecules.

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A method for the stepwise, controlled synthesis of chemical species, particularly peptides, coupled products obtained by the method and the use of these coupled products, e.g. as vaccines

FIELD OF THE INVENTION

The present invention concerns a method for the stepwise, controlled synthesis of chemical species, particularly peptides, using a proteinaceous substance such as a protein as the synthesis substrate.

Peptides are synthesized on amino or carboxylic acid groups of the proteinaceous substrate, either directly or via a linker group which has been attached to amino or carboxylic acid groups or to other functional groups in the substrate. In the case of direct synthesis the method is well-suited for building-up on the proteinaceous substrate a number of peptide chains having the same primary sequence, and to be presented for a given biological or serological system, e.g. for immunization, antibody assay etc.

In the second case the method is well suited for the synthesis of free peptides. In both cases the carboxylic acid groups on the proteinaceous substrate may be used in either unprotected or protected form, or may be functionalized before or after peptide synthesis in a desired manner to allow special chemical or biological properties of the conjugates to be obtained. Furthermore, the synthesis of peptides on the amino or carboxylic acid groups can be followed up by synthesis of another peptide on suitably modified, initially unexploited carboxylic acid, amino or other groups on the substrate or by conjugation to other molecules such as sugars, lipids, etc.

According to the invention, peptide conjugates may be obtained with a high degree of peptide/substrate substitution. The method provides well-defined protein conjugates at a far lower cost than any conventional approach, and it eliminates a number of otherwise time consuming and expensive reactions.

The method is suited, for example, to the preparation of peptide/protein conjugates and free peptides in amounts varying from milligrams to kilograms.

BACKGROUND OF THE INVENTION

Traditional solid-phase peptide synthesis (SPPS) involves the use of an insoluble polymeric support functionalized with an anchoring group to which the growing peptide chain is attached. Coupling reactions and deprotection steps can be carried out in the

same vessel, and unreacted soluble reagents can be removed by simple filtration and washing. This strategy, originally developed by R.B.Merrifield (see, e.g., J.Am.Chem.Soc. 85, 2149 (1963)) is generally far better than synthesis in homogeneous solution with respect to product yield and the amount of work necessary, and for automating the process of synthesis.

The original polymeric support introduced by Merrifield (the "Merrifield resin") is a functionalized cross-linked styrene/divinylbenzene copolymer. It is normally provided in the form of spheres or particles, often with a predominant particle size of 20-80 μm. The functionalization originally preferred was functionalization of the aromatic rings of the copolymer with chloromethyl groups. Subsequently, well over 50 functionalization methods have been described among which Friedel-Crafts reactions to introduce chloromethyl, aminomethyl, and benzhydrylamino groups are the most widely applied [see, e.g., G.Barany et al., Int.J.Peptide Protein Res. 30, 705-739 (1987)]. The Merrifield resin has been used for numerous syntheses and has undergone further development. Reduction of the divinylbenzene content of the polymerization mixture from 2 to 1 % resulted in a resin that swells better in commonly employed solvents such as dichloromethane or dimethylformamide. Good swelling properties of synthetic resins are of great importance, as a small fraction of the functionalized molecules otherwise remains hidden in certain areas of the synthetic substrate such that it is inaccessible for acylation by activated derivatives of hindered amino acids such as threonine and isoleucine.

Regardless of its nature, the purpose of the functionality is to provide an anchoring bond between the support and the C-terminal of the first amino acid which it is desired to couple to the support. Other refinements of the Merrifield technique include incorporation of a bifunctional "linker" group (also called a "spacer" or

"handle" group) between a functionality (e.g. one of the above-mentioned functionalities) on the polymer chain of the support and the C-terminal of the first amino acid to be coupled, the linker being tailored, inter alia, to fulfil certain requirements with regard to the coupling of the first amino acid to the support and/or with regard to the ease with which the completed synthesized peptide chain is cleaved from the support.

In the 1970's, the increasing needs of molecular biology prompted a reappraisal of the SPPS method, and it was evident that the original reaction conditions devised by Merrifield were not necessarily optimal. Consideration of solvation effects within the polymer matrix led to replacement of the original non-polar Merrifield resin by a new, polar dimethylacrylamide/ethylene bisacrylamide/acryloylsarcosine methyl ester copolymer resin. This latter beaded, gelatinous polymer is freely permeated and solvated by a large number of solvents of the dimethylformamide type. Furthermore, dimethylformamide is generally well-suited as a solvent for protected peptides, and is a preferred solvent for many peptide bond formation reactions (see, e.g., R.C.Sheppard, Chemistry in Britain, 402 (1983)).

The above-described types of polymer supports, i.e. the Merrifield and the "polyamide" type, make use of two different types of amino acid protecting groups, and their use thus entails two chemically different methodologies. In the "polyamide" methodology, the conventional acid-labile t-butoxycarbonyl protecting groups are replaced with other, base-labile N-protecting groups such as Fmoc, which is cleaved off within a few seconds upon treatment with secondary amines such as piperidine. At the same time, the benzyl-based side-chain protecting groups employed in the traditional Merrifield methodology are replaced with, e.g., t-butyl-based side-chain protecting groups. The "polyamide" deprotection scheme makes peptide synthesis possible under considerably milder conditions than in the Merrifield strategy.

Useful designs of solid-phase peptide synthesis methods which employ the above described "polyamide" substrate are normally those in which the growing peptide chain is attached to the support during the synthesis by means of an acid- or base-cleavable "linker", e.g. 4-hydroxymethylphenoxyacetic acid, 3-methoxy-4-

hydroxymethyl-hydroxymethylphenoxyacetic acid (both of which are acid-cleavable) or 4-hydroxy-methylbenzoic acid (which is base-cleavable). Thus, the methyl ester functions of the substrate are first reacted with ethylenediamine. The free amino groups in the ethylenediamine units are then acylated with the "linker", after which the first amino acid is coupled to the hydroxymethyl group of the "linker" in the form of an Fmoc-protected amino acid anhydride; the coupling process is catalyzed by DMAP. Alternatively, the first amino acid can be coupled to the support, after packing the latter in suitable columns, wells or other reaction containers, by the reaction of the Fmoc-protected amino acid Pfp ester catalyzed by DMAP.

After establishing the support with the coupled, first, at least N-protected amino acid, the synthesis cycle continues with cleavage of the Fmoc group from the coupled, first amino acid followed by coupling of the next Fmoc-protected amino acid with its carboxyl group suitably activated, e.g. by introducing the Fmoc-protected amino acid in the form of a symmetrical anhydride or an activated ester.

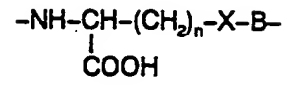
With assembly of the polymer-bound protected, polypeptide chain satisfactorily completed, there follows cleavage of the anchoring linkage. Depending on the chemistry of the original handle/linker and on the cleavage reagent selected, the product from this step can be a C-terminal peptide acid, amide, ester or hydrazide (see, e.g., J.M.Stewart and J.D.Young (1984) in Solid Phase Peptide Synthesis, 2nd edn., Pierce Chemical Co., Rockford IL). Appropriate reagents for cleavage from Merrifield type supports include acids such as neat, anhydrous liquid hydrogen fluoride (HF) [see e.g. W.F.Heat, J.P.Tam and R.B.Merrifield, Int.J.Peptide Protein Res. 28, 498 - 507 (1986)] or trifluoromethanesulfonic acid in trifluoroacetic acid (see e.g. J.P.Tam, W.F.Heath and R.B.Merrifield J.Amer.Chem.Soc. 108, 5242 - 5251 (1986), and references cited therein); and nucleophiles such as ammonia, alkoxide, hydrazine or thiophenoxide in a variety of polar solvents (see e.g. Stewart and Young: vide supra). Reagents for cleavage of peptides from "polyamide" resins include trifluoroacetic acid and the above mentioned nucleophiles (see, e.g., R.Sheppard; vide supra).

Literally thousands of naturally-occurring peptides or their analogues have been synthesized using the original method, and recent developments has led to an increase of several orders of magnitude in the output of synthetic peptides for use in the elucidation of biological proceses (see e.g. Barany et. al. vide supra). One important aspect is the use of synthetic peptides as vaccines, e.g. antibacterial and antiviral vaccines, and vaccines against hormones (see, e.g., E.Lederer, Drugs Exptl.Clin.Res. XII (1986) 429-440). Synthetic peptides being mostly weak immunogens, it is necessary to couple them to carriers and to make use of adjuvants (see e.g. M.Sela, In: Progress in immunology, V, Academic Press Japan Inc., (1983) 13; R.Arnon Pharmac. Ther. 6 (1979) 275; R.Arnon Ann.Rev.Microbiol. 34 (1980) 593; T.M.Shinnik, J.G.Sutcliffe, N.Green and R.A.Lerner Ann.Rev.-Microbiol. 37 (1983) 425). Amongst natural carriers the most frequently used are bovine serum albumin (BSA) (see, e.g., F.Audibert et al. Nature 289 (1981) 593), tetanus toxoid (see, e.g., E.H.Beachey et al., In: J.W.Hadden et.al., eds., Advances in immunopharmacology, 2, Pergamon Press, Oxford and New York (1983) 407), keyhole limpet hemocyanin (KLH) (see, e.g., J.L.Bittle et al. Nature 298 (1982) 30) and purified protein derivative (PPD); synthetic carriers used are poly-L-lysine (see, e.g., E.H.Beachley et al. vide supra; D.N.Possnett, H.McGrath and J.P.Tam J.Biol.Chem. 263 (1988) 1719-1725) or poly-DL-Ala-(poly-L-Lys)(A - L) (see e.g. F.Audibert et al. vide supra)

Glutaraldehyde, which is frequently used as coupling agent between a peptide and a carrier protein has the disadvantage of leading to unspecific coupling and not always giving reproducible results. More specific methods use bifunctional linkers such as the N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide (see e.g. S.Yoshitake et al. J.Biochem. 92 (1982) 1413-1424), N-succinimidyl 3-(2-pyridyldithio)propionate (see, e.g., J.Carlsson, H.Drevin and R.Axen Biochem.J 173 (1978) 723-737) and others (see e.g. PIERCE, Handbook and General Catalog 1985 - 86 (Pierce Chemical Company, P.O.Box 117, Rockford, IL U.S.A.): Chemical Modification, "Double Agents", p. 316-332).

EP-A1-0329994 describes an immunogen and its use in recovering antibodies against hemoglobin A1 C. An immunogenic peptide is coupled to a protein by

means of a spacer A with the formula



wherein X is S or NH and B is an organic residue containing a succinimide group. The peptide is prepared separatley following a known synthesis protocol. It is then coupled to a protein in a convenient way. The method does not comprise stepwise peptide synthesis on a protein and it does not differ essentially from well-known conjugating methods.

According to EP-A2-0175613, a peptide containing the amino acid residues 13 to 24 of the VP1 structural HAV protein is conjugated chemically to a carrier protein. The method of conjugation is the well-known glutaraldehyde method.

The chapter "Synthetic Polypeptides as Antigens" in Laboratory Techniques in Biochemistry and Molecular Biology, vol. 19, 1988, by R.H. Burdon et.al., Elsevier, Amsterdam, pp. 95-103, describes well-known methods of conjugating peptides to proteins. A direct, stepwise peptide synthesis on proteins, however, is not mentioned.

EP-A1-0284492 describes novel peptide fractions which induce the formation of protective antibodies against bovine leukemia virus. A process for the preparation of these peptide fractions and their use, e.g. as vaccines, is also described.

The peptide is prepared separately according to a known synthesis protocol and conjugated chemically to a carrier protein by well-known methods using hetero-bifunctional reagents.

Finally, EP-A2-0243929 concerns polypeptide and protein derivatives and a process for their preparation. The method comprises protein and polypeptide derivatives and salts thereof, which are characterized in that a protein or a polypeptide is conjugated via a selected group to the same or a different protein or polypeptide.

The use of proteinaceous substances, e.g. proteins, as substrates for, e.g., solid-phase peptide synthesis (SPPS), instead of the conventionally employed synthetic and often expensive polymer resin substrates (vide supra), has not been described previously, although such a method is conceptually highly attractive from several points of view. The lack of such a methodology may have many causes but is probably due to a general belief that the proteinaceous substance would be lost during the numerous essential washes with solvents and treatments with reagents. The solvents for SPPS generally include solvents such as dichloromethane and N,N-dimethylformamide, and the reagents often comprise trifluoroacetic acid and piperidine. It is therefore imperative that the proteinaceous substrates in question are of very low solubility or are virtually insoluble in media comprising such solvents/reagents in order to permit the carrying out of a satisfactory number of washing steps. This prerequisite can, of course, be verified by very simple weighing/washing experiments before attempting any actual synthesis, and this constitutes part of the technique of the present invention.

The best-described class of proteins among the common carrier proteins is the serum albumins. Several reviews on albumins have appeared in the past few decades (see, e.g., T.Peters, Jr. Adv.Protein Chemistry 37 (1985) 161 -245). In the earliest separations of plasma proteins, the fraction which remained soluble upon dialysis against water was named albumin, and the precipitated proteins were named globulins. Today, the term albumin is generally taken to mean serum albumin or plasma albumin isolated from man (HSA), cow (BSA) and several other animal species. Albumin's chief traits are those of an acidic, very water-soluble, stable protein. Its isoelectric point when carrying fatty acids is about pH 4.8. Neutral 30% (w/v) solutions are readily prepared and are used in serology. At pH 6-7, albumin is soluble in concentrated aqueous ammonium sulfate solutions (2.1 M) or in aqueous ethanol (40% v/v, at -5°C), but can be precipitated by acidifying to pH 5. Albumin's stability, or resistance to denaturation, has long been recognized. The molecule can withstand heating to 60 °C for 10 hours (in the presence of stabilizers such as caprylic acid). At pH 1-2 the albumin molecule expands and elongates; it can return to its native configuration reversibly. Albumin is relatively unaffected at pH 9-11, but it may undergo deamidation or disulfide interchange. Its resistance to acid conditions and to organic solvents is exploited in a simple

procedure using 1% trichloroacetic acid/ 95% ethanol to isolate albumin from globulins.

Albumins are characterized by having a low number of tryptophan and methionine moieties and a large number of cysteine moieties and of the charged amino acids aspartic acid (41 and 36 in BSA and HSA, respectively), glutamic acid (59 and 62 in BSA and HSA, respectively), lysine (59 in BSA and HSA) and arginine (23 and 24 in BSA and HSA, respectively). The glycine and isoleucine contents are lower than in many other common proteins. Albumins are proteins consisting of a single chain of about 580 residues, cross-linked by 17 disulfide bridges of nine loops - six large and three small - and consist of three alfa-helical domains.

The presence of functional groups such as amino, carboxylic, and disulfide groups has allowed a number of, mainly single step, chemical modifications to be carried out with serum albumin, keyhole limpet hemocyanin and certain other proteins. These modifications include conjugation of peptides to carrier proteins using glutaraldehyde or the above-mentioned bifunctional agents, conjugation of proteins and antibodies with fluorochromes such as isothiocyanate derivatives of fluorescein or rhodamine (see, e.g., J. W. Goding, Monoclonal Antibodies: Principles and Practice, 1983, Academic Press), reduction of disulfide bonds followed by carboxymethylation with iodoacetic acid (see, e.g., C. H. W. Hirs, Reduction and S-Carboxymethylation of Proteins in C. H. W. Hirs (Ed.) Methods in Enzymology XI, Academic Press, N.Y., 1967, pp. 199-203), acylations, reaction with chloramine-T (see, e.g., B. L. Vallee and J. F. Riordan, Ann.Rev.Biochem. 38 (1969) 733). However, peptide synthesis per se, involving the numerous repetitive synthesis steps, using albumins or other proteinaceous macromolecules has not been described.

One aspect of the invention relates to a method for the stepwise, controlled synthesis of chemical species, particularly peptides, the method comprising

(1) taking a proteinaceous substance, the proteinaceous substance conforming to at least one of the following criteria:

A. it is a polypeptide, oligopeptide or protein, as defined by the material comprising:

at least 10 amino acid moieties, of which preferably at least two are different,

preferably at least 20 amino acid moieties, of which preferably at least two are different,

in particular at least 30 amino acid moieties, of which preferably at least two are different,

preferably at least 40 amino acid moieties, of which preferably at least two are different,

especially at least 50 amino acid moieties, at least two of which are different,

B. It comprises, as a significant part of its molecular structure, a polypeptide, oligopeptide, or protein as defined under A, the substance being, e.g., a glycoprotein, a lipoprotein, a protein conjugated with nucleic acids, or a substancecomprising mixtures thereof, e.g. a bacterial cell, a bacterial cell wall fragment, or cell extract,

(2) subjecting the proteinaceous substance to a chemical reaction, in a reaction medium, with a reactant selected from:

(i) an amino acid which is activated at the C-terminal carboxy group and at least N-terminal protected and in which any side-chain functionalities which can give rise to undesired reactions optionally are protected,

(ii) a peptide which is activated at the C-terminal carboxy group and at least N-terminal protected and in which any side-chain functionalities which can give rise to undesired reactions optionally are protected,

(iii) any other molecule which is reactive towards available functionalities on the proteinaceous substance so as to form a bond between the proteinaceous substance and the molecule, the molecule being, e.g, an amino sugar, a carboxylated sugar, an activated acid, an amine, an aldehyde or a ketone,

(3) removing excess reactant and reaction medium from the reaction product by filtration through a filter which allows passage of the reactant and reaction media, but substantially retains the reaction product,

(4) washing the reaction product with a washing medium, and removing the washing medium from the washed reaction product by filtration through a filter which allows passage of the washing medium, but substantially retains the reaction product, and

(5) repeating the cycle comprising steps as defined under (2)-(4) until the desired chemical species has been obtained, this repetition of steps as defined under (2)-(4) being optional when the reactant is an amino acid or a peptide as defined under (2),

the reactant in the first step optionally being selected from:

1) reactants which, when coupled to the proteinaceous substance, provide a linker group useful as reaction target for a reactant selected from those defined under (2) (i), (2) (ii) and (2) (iii), the linker group being selected from

(a) groups which are capable of being cleaved under conditions which substantially do not deteriorate the reaction product formed on the linker group as a result of the treatment with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and

(b) groups which are adapted to provide a spacing (molecular distance) between the proteinaceous substance and the reaction product formed on the linker group as a result of the treatment with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and/or special physicochemical properties such as an increase or decrease of the solubility, in aqueous and/or organic solvents, of the proteinaceous substance carrying the linker group and the reaction product formed on the linker group as a result of the treatment with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and

2) reactants which permanently or reversibly blocking selected functional groups, in particular carboxylic acid groups, in the proteinaceous substance, such as primary or secondary amines, e.g. dialkylamines, e.g. diethylamine, and lower alkanolamines such as 2-ethanolamine, optionally combined with an activating reagent such as diisopropyl carbodiimide, dicyclohexylcarbodiimide or BOP reagent.

As will be understood, the proteinaceous substance is either a protein or a molecule a significant proportion of which is a protein. The protein (or the protein part of another molecule) is used directly as the substrate for the synthesis, the synthesis either being carried out directly on a native functionality present on the protein, or on a functionality introduced by modification of such a native functionality. Such functionalities comprise amino, carboxyl, hydroxy, and thiol. The gist of the present invention is to introduce, on a specific functionality on the protein, a coupled group via a specific functionality on the coupling reagent, thereby coupling a desired chemical entity to the protein in a functionality-specific manner, as

contrasted to the known conjugation of a molecule to a protein by use of high concentrations of bifunctional reagents. Thus, the method permits highly specific attachment of e.g. protected amino acids, peptides and other reactants. The method has been found to be very effective and to permit the production of high yields of synthesis product. Furthermore, the method permits highly specific attachment of several different peptides or reactants by controlled use of different functionalities on the protein as explained in the following.

As mentioned above, the reactant may be an activated amino acid or an activated peptide, or the reactant may, in principle, be any molecule which can react with a functionality (native or provided) on the protein, e.g., linker molecules for establishing cleavable linking and/or spacing linking for later reaction cycles, or active molecules such as therapeutically active molecules, e.g. molecules of a medicament, such as, e.g. a steroid.

One of the critical features of the invention is use of the proteinaceous substance as the "solid-phase" substrate for the synthesis. This function of the proteinaceous substance is conditioned by the substance being capable of being retained on a filter during the steps of reagent removal and washing, and preferably during the coupling steps. Most proteins are substantially insoluble in a large number of solvents useful in these steps of the method of the invention, such as DCM, DMF, piperidine/DMF, ethyl acetate, etc., and this substantial insolubility is thus utilized according to the invention to allow separation of the reactants from the reaction products using the proteinaceous substance itself as the insoluble substrate.

On the other hand, most proteins are of a molecular size such that they may be retained, even in dissolved state, on suitable membrane types allowing passage of smaller molecules such as the reactants. This makes it possible to perform the method of the invention in a special manner, using, as reaction and washing media, e.g., water or aqueous media in which the proteinaceous substance is soluble, and carrying out separation steps through membranes or dialysis tubes or the like which will retain the proteinaceous reaction product, but will allow passage of reactants and solvents. The "filters" to be used in this particular embodiment should be selected in accordance with the size of the proteinaceous substance.

Examples of suitable molecular weight exclusion limits are, e.g., from 500 to 300,000, such as, e.g., 1000, 5000, 10,000, 30,000, 50,000, 100,000. The exclusion limit employed for a given proteinaceous substance will, of course, be chosen in accordance with the molecular weight of the substance and the reactants.

It has been found that it is often important for the succesful coupling of, e.g, activated amino acids to the proteinaceous substance that certain functional groups, in particular carboxyl groups, in the proteinaceous substance, are "blocked" with suitable reagents in the first step. The "blocking" reagents are typically amines, such as primary or secondary amines. At present, it is contemplated that the effect of the "blocking" is either ascribable to raising the pH in the microenvironment adjacent to the proteinaceous substance, in which case addition of non-reacting base such a trialkylamine such as triethylamine should produce a similar effect, or ascribable to a certain "denaturing" involving modification of the conformation of the protein.

In very interesting embodiments of the invention, the protein part of the proteinaceous substance is selected from naturally occurring proteins and modified naturally occurring proteins, such as:

egg white, albumins, in particular serum albumins (SA), including human serum albumin (HSA) and bovine serum albumin (BSA), parvoalbumin, α-lactalbumin, ovalbumin, gelatin, lactoferrin, ß-lactoglobulin, histones, keratins, keyhole limpet hemocyanin (KLH), tetanous toxoid (TNT), cholera toxin, hirudin, purified protein derivative (PPD), prolamins, hemocyanin, globins, hemoglobins, including methemoglobin, orosomucoid, transferrin, ferritin, apoferritin, ferritoxin, ceruloplasmin, haptoglobin, fibrinogen, fibrin, blood coagulation factors, Cohn blood fractions, agglutinins, plasmin, avidin, globulins, including α-globulins, e.g., α_1-globulin and α_2-globulin, such as α_2-macroglobulin, ß-globulin, $ß_2$-microglobulin, ⊖-globulin, thyroglobulin, Cold insoluble globulin, lipoproteins, including α_1-lipoprotein, α_2-lipoprotein, $ß_1$-lipoprotein and apolipoprotein, glycoproteins, including α_1-glycoprotein, enzymes, including lactic dehydrogenase, phosphorylase A and B, lysozyme, pepsin, carbonic anhydrase, urease, trypsinogen, trypsin, trypsin inhibitor, chy

motrypsin, elastase, ribonuclease, amylases, including α-amylase and ß-amylase, cholinesterase, peroxidases, catalases, alcohol dehydrogenase, cytochrome C, cytochrome C reduktase, reticulocyte lysate, fetuin, asialofetuin, C-reactive protein, α-foetoprotein, actin, elastin, myosin, tropomyosin, troponin, mitellogenin, myoglobin, collagen, protamine, lectin, including Con A, protein A, casein, glutens, zeins, yeast cell protein extract, bacterial cell protein extract, plant cell protein extract, animal cell protein extract and single cell protein,

or constitutes a part or fragment of any of the above-mentioned proteins.

Many of these proteins are available at low cost and in suitable purity.

One of the most important uses of the method of the invention is for producing peptide moieties bound to the proteinaceous substance. In this case, the reactant is an amino acid as defined under (2) (i) or a peptide as defined under (2) (ii) above, and the cycle comprising steps (2)-(4) is performed two or more times with either such an amino acid or such a peptide in each step, the amino acid being the same or different in each step, and the peptide being the same or different in each step, to build up peptide chains on amino groups on the proteinaceous substance, the following steps being performed after each respective step (4):

(4a) deprotection of the N-terminal of the last-coupled amino acid or peptide prior to each new coupling reaction, the deprotection being performed by treatment with a deprotecting agent adapted to cleave off the protection group in question,

(4b) removing excess deprotecting agent and deprotecting media from the deprotected reaction product by filtration through a filter which allows passage of the deprotecting agent and deprotecting media, but substantially retains the deprotected reaction product, and

(4c) washing the deprotected reaction product with a washing medium, and removing the washing medium from the washed deprotected reaction product

by filtration through a filter which allows passage of the washing medium, but substantially retains the deprotected reaction product,

optionally with final removal of any side-chain deprotecting groups present by

(4d) treatment with a side-chain deprotecting agent and removal of excess side-chain deprotecting agent and side-chain deprotecting media from the side-chain deprotected reaction product by filtration through a filter which allows passage of the side-chain deprotecting agent and side-chain deprotecting media, but substantially retains the side-chain deprotected reaction product, and

(4e) washing the side-chain deprotected reaction product with a washing medium, and removing the washing medium from the washed, side-chain deprotected reaction product by filtration through a filter which allows passage of the washing medium, but substantially retains the side-chain deprotected reaction product.

As mentioned above, amino groups of the proteinaceous substance may be first reacted with a linker reactant which, when coupled to the proteinaceous substance, provides a linker group useful as reaction target for an amino acid as defined under (2) (i) or a peptide as defined under (2) (ii) above, the linker group being a group which is capable of being cleaved under conditions which substantially do not deteriorate the peptide formed on the linker group, the method optionally further comprising:

cleaving the linker group by providing conditions under which the group is cleaved, subsequently separating the peptide reaction product, formed on the linker group, from the proteinaceous substance by filtration through a filter which allows passage of the peptide product and the reaction media, but substantially retains the proteinaceous substance, and recovering the peptide product from the filtrate.

The proteinaceous substance may be subjected, in a first step, to a treatment or treatments converting functional groups other than amino groups to linker groups useful as reaction target for a reactant selected from those defined under (2) (i), (2) (ii) and (2) (iii), the linker groups being selected from:

(a) groups which are capable of being cleaved under conditions which substantially do not deteriorate the reaction product formed on the linker group as a result of the treatment with one or more reactant or coupling reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and

(b) groups which are adapted to provide a spacing (molecular distance) between the proteinaceous substance and the reaction product formed on the linker group as a result of the treatment with one or more reactant or coupling reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and/or special physicochemical properties such as an increase or decrease of the solubility, in aqueous and/or organic solvents, of the proteinaceous substance carrying the linker group and the reaction product formed on the linker group as a result of the treatment with one or more reactant or coupling reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii).

The groups thus converted may be, e.g.:

carboxylic acid groups, which are converted into an amino-terminated group by treatment with a bifunctional compound, such as a diamine, e.g. ethylenediamine, capable of forming an amide bond with the carboxylic acid group and providing a free amino target group,

hydroxy groups which, e.g., are reacted with an α-haloacid to form a carboxy-terminated group which is subsequently converted into an amino-terminated group by treatment with a bifunctional compound, such as a diamine, e.g. ethylenediamine, capable of forming an amide bond with the carboxylic acid group and providing a free amino target group,

disulfide bonds, which are first reduced to SH groups and then reacted with an α-haloacid to form a carboxy-terminated group which is subsequently converted into an amino-terminated group by treatment with a bifunctional compound, such as a diamine, e.g. ethylenediamine, capable of forming an amide bond with the carboxylic acid group and providing a free amino target group.

One of most fascinating possibilities of the invention is that this selective rendering available of a new population of groups of a functionality makes it possible to perform one or several cycles as defined above on a species of functional group on the proteinaceous substance, such as amino, the reaction product, such as a peptide, from the cycle or the last of the cycles being left in at least N-terminal protected form, preferably in fully protected form including both side chain protection and N-terminal protection, after which treatment or treatments of another species of functional group is/are performed to convert the species into a target group is/are performed, and the target group thus made available is subjected to reaction with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii) above throughout one or several cycles as described above. This makes it possible to produce, in a controlled manner, a wide variety of coupled products with two or more different groups coupled to the proteinaceous substance, such as described in greater detail below.

The method of the invention makes it possible to prepare a large number of coupled products comprising a proteinaceous substance conforming to at least one of the following criteria:

A. it is a polypeptide, oligopeptide or protein, as defined by the material comprising:

preferably at least 10 amino acid moieties, of which preferably at least two are different,

preferably at least 20 amino acid moieties, of which preferably at least two are different,

in particular at least 30 amino acid moieties, of which preferably at least two are different,

preferably at least 40 amino acid moieties, of which preferably at least two are different,

especially at least 50 amino acid moieties, at least two of which are different,

B. It comprises, as a significant part of its molecular structure, a polypeptide, oligopeptide, or protein as defined under A, the substance being, e.g., a glycoprotein, a lipoprotein, a protein conjugated with nucleic acids, or a substance comprising mixtures thereof, e.g. a bacterial cell, wall fragment, or a cell extract,

the substance carrying a group or a multitude of groups coupled thereto via a bond formed with a native or modified (e.g. via the introduction of a linker) functional group on the polypeptide, oligopeptide or protein part of the proteinaceous substance, the group or groups comprising a species selected from:

amino acid groups which are protected with a protecting group other than unsubstituted amide and other than lower alkyl (C_1–C_4) ester,

peptide groups, and

groups which can be prepared by reaction between native or modified (e.g. via the introduction of a linker) functional groups on the polypeptide, oligopeptide or protein part of the proteinaceous substance and molecules other than amino acid or peptide molecules which are reactive towards such functional groups to form a bond, in particular an amide bond or an ester bond, between the proteinaceous substance and the molecule in question, the molecule being, e.g, an amino sugar, a carboxylated sugar, an activated acid, an amine, an aldehyde or a ketone. It is believed that these classes of products are novel per se, and thus, they constitute an aspect of the invention.

The multitude of coupled groups may be a number of groups less than or equal to the number of the relevant functional groups on the polypeptide, oligopeptide or protein part of the proteinaceous substance. The extent of coupling may be controlled in each particular case by adjustment of the reaction conditions, such as the concentration of the reactant, the duration of the treatment, etc.

A particularly interesting coupled product is a product carrying a multitude of groups coupled thereto, the groups comprising at least two different species selected from:

amino acid groups which are protected with a protection group other than unsubstituted amide and other than lower alkyl (C_1–C_4) ester,

peptide groups, and

groups which can be prepared by reaction between native or modified (e.g. via the introduction of a linker) functional groups on the polypeptide, oligopeptide or protein part of the proteinaceous substance and molecules other than amino acid or peptide molecules which are reactive towards such functional groups to form a bond, in particular an amide bond or an ester bond, between the proteinaceous substance and the molecule in question, the molecule being, e.g, an amino sugar, a carboxylated sugar, an activated acid, an amine, an aldehyde or a ketone.

An interesting coupled product is one in which the coupled group or groups is/are a peptide group or peptide groups selected from one or several peptide species, in particular where the peptide group or groups is/are selected from the group consisting of peptide hormones, and precursors, fragments, derivatives, antagonists and agonists thereof, the peptide hormones being, in particular, peptide hormones selected from the group consisting of

ß–endorphin,
ß–lipotropin,
Θ–endorphin,
gastrin releasing peptide,

growth hormone releasing factor,
neurotensin,
α-melanocyte stimulating hormone,
adrenocorticotropic hormone,
vasopressin,
angiotensin,
substance P,
dynorphin,
bradykinin,
luteinising hormone releasing hormone,
parathyroid hormone,
atrial natriuretic factor,
atriopeptin,
calcitonin,
calcitonin gene related peptide,
dermorphin,
kyotorpin,
auriculin,
β-casomorphin,
tachykinin,
bombesin,
caerulein,
chorionic gonadotropin,
cholecystokinin,
casomorphin,
corticotropin,
corticotropin releasing factor,
corticotropin inhibiting peptide,
somatostatin,
eukephalin,
gastric inhibitory peptide,
gastrin,
gastrin related peptide,
thyrotropin releasing hormone,

glucacon,
growth hormone,
growth hormone releasing factor,
thyroid stimulating hormone,
prolactin,
neurokinin A and B,
neuromedin B, C, K, L, and U,
neuropeptide Y,
neurotensin,
oxytocin,
pancreastatin,
vasoactive intestinal peptide,
secretin,
galanin,
pancreatic polypeptide,
thymosin and
leucokinin.

Another interesting coupled product is one in which the peptide group or groups is/are selected from the group consisting of peptide growth factors, and precursors, fragments, derivatives, antagonists and agonists thereof, the peptide growth factors being, in particular, peptide growth factors selected from the group consisting of

fibroblast growth factor,
transforming growth factor α,
transforming growth factor ß I and II,
insulin,
cachetin,
epidermal growth factor,
bursin,
insulin-like growth factor I,
insulin-like growth factor II,

inhibin,
liver cell growth factor (Gly His Lys),
nerve growth factor,
platelet derived growth factor,
tuftsin, and
interleukins.

Another interesting coupled product is one in which the peptide group or groups is/are selected from the group consisting of synthetic fragments of proteins with defined biological effect, and derivatives, fragments and analogues thereof, the proteins being, in particular, proteins selected from the group consisting of

fibronectin cell attachment sequence (Gly Asp Arg)
fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
leucine zipper sequence of, e.g., c-fos or c-jun,
helix-turn helix DNA binding sequence,
a protease inhibitor,
a kinase inhibitor,
a muramyl peptide,
zinc finger sequence,
B or T cell activating sequence,
nuclear translocation signal such as Lys Lys Lys Arg Lys,memrane translocation signal and glycosylation sequence (Asn X Ser/Thr),
homeobox sequences,

and any combination of these sequences with each other or with hormones, growth factors or derivatives thereof cited above.

Particularly interesting products of the above type are products in which proteinaceous substance moiety is an albumin, especially human serum albumin or bovine serum albumin.

Other very interesting coupled products are products in which the protein is purified protein derivative (PPD) or tetanus toxoid (TNT).

Specifically intersting classes of coupled products are:

A coupled product which is human serum albumin (HSA) coupled to a hormone, and in particular being selected from the group consisting of

HSA-β-endorphin,
HSA-β-lipotropin,
HSA-Θ-endorphin,
HSA-gastrin releasing peptide,
HSA-growth hormone releasing factor,
HSA-neurotensin,
HSA-α-melanocyte stimulating hormone,
HSA-adrenocorticotropic hormone,
HSA-vasopressin,
HSA-angiotensin,
HSA-substance P,
HSA-dynorphin,
HSA-bradykinin,
HSA-luteinising hormone releasing hormone,
HSA-parathyroid hormone,
HSA-atrial natriuretic factor,
HSA-atriopeptin,
HSA-calcitonin,
HSA-calcitonin gene related peptide,
HSA-dermorphin,
HSA-kyotorpin,
HSA-auriculin,
HSA-β-casomorphin,
HSA-tachykinin,
HSA-bombesin,
HSA-caerulein,
HSA-chorionic gonadotropin,
HSA-cholecystokinin,
HSA-casomorphin,

HSA-corticotropin,
HSA-corticotropin releasing factor,
HSA-corticotropin inhibiting peptide,
HSA-somatostatin,
HSA-eukephalin,
HSA-gastric inhibitory peptide,
HSA-gastrin,
HSA-gastrin related peptide,
HSA-thyrotropin releasing hormone,
HSA-glucacon,
HSA-growth hormone,
HSA-growth hormone releasing factor,
HSA-thyroid stimulating hormone,
HSA-prolactin,
HSA-neurokinin A and B,
HSA-neuromedin B, C, K, L, and U,
HSA-neuropeptide Y,
HSA-neurotensin,
HSA-oxytocin,
HSA-pancreastatin,
HSA-vasoactive intestinal peptide,
HSA-secretin,
HSA-galanin,
HSA-pancreatic polypeptide,
HSA-thymosin and
HSA-leucokinin,

the hyphen designating a coupling amide bond between a functional group on HSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the HSA molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of

the relevant functional groups on the HSA molecule,

and the corresponding coupled products where the peptide species coupled to the HSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone;

a coupled product which is human serum albumin (HSA) coupled to a peptide growth factor, and in particular being selected from the group consisting of:

HSA–fibroblast growth factor,
HSA–transforming growth factor α,
HSA–transforming growth factor ß I and II,
HSA–insulin,
HSA–cachetin,
HSA–epidermal growth factor,
HSA–bursin,
HSA–insulin–like growth factor I,
HSA–insulin–like growth factor II,
HSA–inhibin,
HSA–liver cell growth factor (Gly His Lys),
HSA–nerve growth factor,
HSA–platelet derived growth factor,
HSA–tuftsin and
HSA–interleukins,

the hyphen designating a coupling amide bond between a functional group in HSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide growth factor in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the HSA molecule carrying one or, preferably, a multitude of the growth factor groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the HSA molecule,

and the corresponding coupled products where the peptide species coupled to the HSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the growth factor;

a coupled product which is human serum albumin (HSA) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, the coupled product being selected from the group consisting of

HSA-fibronectin cell attachment sequence (Gly Asp Arg)
HSA-fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
HSA-leucine zipper sequence of e.g. c-fos or c-jun,
HSA-helix-turn helix DNA binding sequence,
HSA-protease inhibitor,
HSA-kinase inhibitor,
HSA-muramyl peptide,
HSA-zinc finger sequence,
HSA-B or T cell activating sequence,
HSA-nuclear translocation signal such as Lys Lys Lys Arg Lys,
HSA-memrane translocation signal,
HSA-glycosylation sequence (Asn X Ser/Thr), and
HSA-muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in HSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the HSA molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the HSA molecule,

and the corresponding coupled products where the coupled peptide is a derivative, fragment or analog thereof;

a coupled product which is bovine serum albumin (BSA) coupled to a hormone, and in particular being selected from the group consisting of:

BSA-ß-endorphin,
BSA-ß-lipotropin,
BSA-Ø-endorphin,
BSA-gastrin releasing peptide,
BSA-growth hormone releasing factor,
BSA-neurotensin,
BSA-α-melanocyte stimulating hormone,
BSA-adrenocorticotropic hormone,
BSA-vasopressin,
BSA-angiotensin,
BSA-substance P,
BSA-dynorphin,
BSA-bradykinin,
BSA-luteinising hormone releasing hormone,
BSA-parathyroid hormone,
BSA-atrial natriuretic factor,
BSA-atriopeptin,
BSA-calcitonin,
BSA-calcitonin gene related peptide,
BSA-dermorphin,
BSA-kyotorpin,
BSA-auriculin,
BSA-ß-casomorphin,
BSA-tachykinin,
BSA-bombesin,
BSA-caerulein,
BSA-chorionic gonadotropin,
BSA-cholecystokinin,
BSA-casomorphin,
BSA-corticotropin,
BSA-corticotropin releasing factor,

BSA–corticotropin inhibiting peptide,
BSA–somatostatin,
BSA–eukephalin,
BSA–gastric inhibitory peptide,
BSA–gastrin,
BSA–gastrin related peptide,
BSA–thyrotropin releasing hormone,
BSA–glucacon,
BSA–growth hormone,
BSA–growth hormone releasing factor,
BSA–thyroid stimulating hormone,
BSA–prolactin,
BSA–neurokinin A and B,
BSA–neuromedin B, C, K, L, and U,
BSA–neuropeptide Y,
BSA–neurotensin,
BSA–oxytocin,
BSA–pancreastatin,
BSA–vasoactive intestinal peptide,
BSA–secretin,
BSA–galanin,
BSA–pancreatic polypeptide,
BSA–thymosin and
BSA–leucokinin,

the hyphen designating a coupling amide bond between a functional group in BSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the BSA molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the BSA molecule,

and the corresponding coupled products where the peptide species coupled to the BSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone;

a coupled product which is bovine serum albumin (BSA) coupled to a peptide growth factor and in particular being selected from the group consisting of:

BSA-fibroblast growth factor,
BSA-transforming growth factor α,
BSA-transforming growth factor β I and II,
BSA-insulin,
BSA-cachetin,
BSA-epidermal growth factor,
BSA-bursin,
BSA-insulin-like growth factor I,
BSA-insulin-like growth factor II,
BSA-inhibin,
BSA-liver cell growth factor (Gly His Lys),
BSA-nerve growth factor,
BSA-platelet derived growth factor,
BSA-tuftsin and
BSA-interleukins,

the hyphen designating a coupling amide bond between a functional group in BSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the BSA molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the BSA molecule,

and the corresponding coupled products where the peptide species coupled to the HSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or

an agonist) of the growth factor;

a coupled product which is bovine serum albumin (BSA) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, and in particular being selected from the group consisting of:

BSA–fibronectin cell attachment sequence (Gly Asp Arg)
BSA–fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
BSA–leucine zipper sequence of e.g. c–fos or c–jun,
BSA–helix–turn helix DNA binding sequence,
BSA–protease inhibitor,
BSA–kinase inhibitor,
BSA–muramyl peptide,
BSA–zinc finger sequence,
BSA–B or T cell activating sequence,
BSA–nuclear translocation signal such as Lys Lys Lys Arg Lys,
BSA–memrane translocation signal,
BSA–glycosylation sequence (Asn X Ser/Thr), and
BSA–muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in BSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), and a functional group of the synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the BSA molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the BSA molecule,

and the corresponding coupled product where the peptide is a derivative, fragment or analogue thereof;

a coupled product which is purified protein derivative (PPD) coupled to a hormone, and in particular being selected from the group consisting of:

PPD–ß–endorphin,

PPD–ß–lipotropin,

PPD–☺–endorphin,

PPD–gastrin releasing peptide,

PPD–growth hormone releasing factor,

PPD–neurotensin,

PPD–α–melanocyte stimulating hormone,

PPD–adrenocorticotropic hormone,

PPD–vasopressin,

PPD–angiotensin,

PPD–substance P,

PPD–dynorphin,

PPD–bradykinin,

PPD–luteinising hormone releasing hormone,

PPD–parathyroid hormone,

PPD–atrial natriuretic factor,

PPD–atriopeptin,

PPD–calcitonin,

PPD–calcitonin gene related peptide,

PPD–dermorphin,

PPD–kyotorpin,

PPD–auriculin,

PPD–ß–casomorphin,

PPD–tachykinin,

PPD–bombesin,

PPD–caerulein,

PPD–chorionic gonadotropin,

PPD–cholecystokinin,

PPD–casomorphin,

PPD–corticotropin,

PPD–corticotropin releasing factor,

PPD–corticotropin inhibiting peptide,

PPD–somatostatin,
PPD–eukephalin,
PPD–gastric inhibitory peptide,
PPD–gastrin,
PPD–gastrin related peptide,
PPD–thyrotropin releasing hormone,
PPD–glucacon,
PPD–growth hormone,
PPD–growth hormone releasing factor,
PPD–thyroid stimulating hormone,
PPD–prolactin,
PPD–neurokinin A and B,
PPD–neuromedin B, C, K, L, and U,
PPD–neuropeptide Y,
PPD–neurotensin,
PPD–oxytocin,
PPD–pancreastatin,
PPD–vasoactive intestinal peptide,
PPD–secretin,
PPD–galanin,
PPD–pancreatic polypeptide,
PPD–thymosin and
PPD–leucokinin,

the hyphen designating a coupling amide bond between a functional group in PPD (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the PPD molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the PPD molecule,

and the corresponding coupled products where the peptide species coupled to the PPD is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone;

a coupled product which is purified protein derivative (PPD) coupled to a peptide growth factor, and in particular being selected from the group consisting of:

PPD–fibroblast growth factor,
PPD–transforming growth factor α,
PPD–transforming growth factor β I and II,
PPD–insulin,
PPD–cachetin,
PPD–epidermal growth factor,
PPD–bursin,
PPD–insulin–like growth factor I,
PPD–insulin–like growth factor II,
PPD–Inhibin,
PPD–liver cell growth factor (Gly His Lys),
PPD–nerve growth factor,
PPD–platelet derived growth factor,
PPD–tuftsin and
PPD–interleukins,

the hyphen designating a coupling amide bond between a functional group in PPD (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the PPD molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the PPD molecule,

and the corresponding coupled products where the peptide species coupled to the PPD is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or

an agonist) of the growth factor;

a coupled product which is purified protein derivative (PPD) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, and in particular being selected from the group consisting of:

PPD–fibronectin cell attachment sequence (Gly Asp Arg)
PPD–fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
PPD–leucine zipper sequence of e.g. c–fos or c–jun,
PPD–helix–turn helix DNA binding sequence,
PPD–protease inhibitor,
PPD–kinase inhibitor,
PPD–muramyl peptide,
PPD–zinc finger sequence,
PPD–B or T cell activating sequence,
PPD–nuclear translocation signal such as Lys Lys Lys Arg Lys,
PPD–membrane translocation signal,
PPD–glycosylation sequence (Asn X Ser/Thr), and
PPD–muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in PPD (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the PPD molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the PPD molecule,

and the corresponding coupled products where the peptide is a derivative, fragment, or analog thereof;

a coupled product which is tetanus toxoid (TNT) coupled to a hormone, and in particular being selected from the group consisting of:

TNT-β-endorphin,
TNT-β-lipotropin,
TNT-Θ-endorphin,
TNT-gastrin releasing peptide,
TNT-growth hormone releasing factor,
TNT-neurotensin,
TNT-α-melanocyte stimulating hormone,
TNT-adrenocorticotropic hormone,
TNT-vasopressin,
TNT-angiotensin,
TNT-substance P,
TNT-dynorphin,
TNT-bradykinin,
TNT-luteinising hormone releasing hormone,
TNT-parathyroid hormone,
TNT-atrial natriuretic factor,
TNT-atriopeptin,
TNT-calcitonin,
TNT-calcitonin gene related peptide,
TNT-dermorphin,
TNT-kyotorpin,
TNT-auriculin,
TNT-β-casomorphin,
TNT-tachykinin,
TNT-bombesin,
TNT-caerulein,
TNT-chorionic gonadotropin,
TNT-cholecystokinin,
TNT-casomorphin,
TNT-corticotropin,
TNT-corticotropin releasing factor,
TNT-corticotropin inhibiting peptide,
TNT-somatostatin,
TNT-eukephalin,

TNT-gastric inhibitory peptide,
TNT-gastrin,
TNT-gastrin related peptide,
TNT-thyrotropin releasing hormone,
TNT-glucacon,
TNT-growth hormone,
TNT-growth hormone releasing factor,
TNT-thyroid stimulating hormone,
TNT-prolactin,
TNT-neurokinin A and B,
TNT-neuromedin B, C, K, L, and U,
TNT-neuropeptide Y,
TNT-neurotensin,
TNT-oxytocin,
TNT-pancreastatin,
TNT-vasoactive intestinal peptide,
TNT-secretin,
TNT-galanin,
TNT-pancreatic polypeptide,
TNT-thymosin and
TNT-leucokinin,

the hyphen designating a coupling amide bond between a functional group in TNT (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the TNT molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the TNT molecule,

and and the corresponding coupled products where the peptide species coupled to the TNT is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone;

a coupled product which is tetanus toxoid (TNT) coupled to a peptide growth factor, and in particular being selected from the group consisting of:

TNT-fibroblast growth factor,
TNT-transforming growth factor α,
TNT-transforming growth factor β I and II,
TNT-insulin,
TNT-cachetin,
TNT-epidermal growth factor,
TNT-bursin,
TNT-insulin-like growth factor I,
TNT-insulin-like growth factor II,
TNT-inhibin,
TNT-liver cell growth factor (Gly His Lys),
TNT-nerve growth factor,
TNT-platelet derived growth factor,
TNT-tuftsin and
TNT-interleukins,

the hyphen designating a coupling amide bond between a functional group in TNT (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the TNT molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the TNT molecule,

and the corresponding coupled products where the peptide species coupled to the TNT is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the growth factor;

a coupled product which is tetanus toxoid (TNT) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, and in particular being

selected from the group consisting of:

TNT-fibronectin cell attachment sequence (Gly Asp Arg)
TNT-fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
TNT-leucine zipper sequence of e.g. c-fos or c-jun,
TNT-helix-turn helix DNA binding sequence,
TNT-protease inhibitor,
TNT-kinase inhibitor,
TNT-muramyl peptide,
TNT-zinc finger sequence,
TNT-B or T cell activating sequence,
TNT-nuclear translocation signal such as Lys Lys Lys Arg Lys,
TNT-memrane translocation signal,
TNT-glycosylation sequence (Asn X Ser/Thr), and
TNT-muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in TNT (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the TNT molecule carrying one or, preferably, a multitude of the synthetic fragment groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the TNT molecule,

and the corresponding coupled products where the peptide is a derivative, fragment or analogue thereof.

In one aspect of the invention, the above-mentioned "polyamide"-type or Merrifield-type reagent strategies, or any other solid-phase peptide synthesis reagent strategy using Fmoc, Boc or any other alfa-amino protecting group or side-chain protecting group, may be employed using albumins or other proteinaceous substances as solution- or solid-phase substrates to synthesize peptides in a free state or attached (conjugated) to the proteinaceous macromolecular substrate. Any

appropriate kind of chemical method for further substitution of the conjugate product with other functions such as lipid functionalities, alcohol groups, etc., may be used before or after peptide synthesis.

One aspect of a method for solid-phase peptide-protein synthesis according to the invention affords peptides directly attached to amino groups of the protein. Using the above-described "polyamide" reagent strategy, after coupling of the first amino acid, piperidine dissolved in DMF is used as base for cleavage of the Fmoc group (i.e. deprotection) from this and from subsequent amino acids. Second, third, etc. amino acids to be coupled are introduced in the form of Fmoc-protected amino acid O-Dhbt or O-Pfp esters or Fmoc-protected amino acids activated with dicyclohexylcarbodiimide, BOP-reagent or a similar activation agent. The Dhbt-ester derivatives have the great advantage that the liberated Dhbt-OH reacts with remaining amino groups present with the formation of the strongly yellow-coloured anion Dhbt-O^{-} which disappears gradually as the amino groups become acylated. [A detailed description of the chemistry involved in this "Dhbt indicator" reaction is given by Atherton et al. (J.Chem.Soc. Perkin Trans. 1 (1988) 2887)]. After cleavage of the Fmoc group from the last-coupled amino acid by treatment with base, e.g. piperidine, it is important that any remaining base is removed by a number of carefully performed washings before introduction of the next amino acid to be coupled. After coupling, excess activated and at least N-protected amino acid must be removed by a number of washings, as remaining amino acid otherwise may cause double coupling of the same amino acid on addition of the piperidine. After completion of the desired peptide sequence side-chain protecting groups are removed by treatment with aqueous trifluoroacetic acid. During this treatment the entire peptide-protein product dissolves in the reagent and is recovered by addition of ether or simple evaporation of the solvent. The peptide-protein product may purified by standard techniques as gelfiltration or dialysis.

In the above-mentioned preferred aspect the amino groups of the macromolecule may be substituted with a non-cleavable linker, such 6-aminohexanoic acid or any other suitable linker, attached e.g. by the carboxylic group of the aminohexanoic acid to the amino groups of the macromolecule and the amino group of the aminohexanoic acid then used for peptide synthesis. Carboxylic acid groups may

be protected e.g. by transformation into a diethylamido group by treatment with diethylamine in the presence of dicyclohexylcarbodiimide as activating agent.

In a further preferred aspect of a method for solid-phase peptide-protein synthesis according to the invention, a cleavable linker group is coupled to the amino groups of the protein. The above-mentioned Fmoc-strategy or any other solid-phase or solution chemistry method may be used. Using the "polyamide" reagent strategy the above-mentioned Fmoc-protected amino acid anhydride/DMAP strategy is employed for coupling of the first amino acid, and piperidine dissolved in DMF is used as base for cleavage of the Fmoc group (i.e. deprotection) from this and the subsequent amino acids. Second, third, etc. amino acids to be coupled are introduced in the form of Fmoc-protected amino acid ester as described above. After completion of the desired peptide sequence, treament with triflouroacetic acid causes cleavage of the side-chain protecting groups and cleavage of the peptide from the protein support. During this treatment the mixture of peptide and protein dissolves in the reagent and the products are recovered by addition of ether or simple evaporation of the solvent. The peptide is separated from the protein carrier by conventional gel filtration or dialysis.

In yet another preferred aspect, carboxylic acid groups may be protected, e.g. by diethyla midation with diethylamine/dicyclohexylcarbodiimide.

In another aspect, a protein substrate is used, in which disulfide groups are reduced to thiol groups followed by treatment with iodoacetic acid to give a S-carboxymethylated protein. Peptide synthesis may then be performed according to the invention.

In a further aspect, a proteinaceous substrate is used in which amino and/or carboxylic groups are reversibly protected followed by conversion of other functional groups such as -OH (Ser, Tyr, Thr, etc.), -SH (Cys, etc.), guanidino (Arg, etc) or other groups in the substrate into amino or carboxylic groups which can then be used for stepwise solid-phase synthesis.

In a further aspect, the above mentioned aspects are used in combination to build up several different peptide chains with or without biological activity, such as hormones, growth factors, cytokines, cell attachment sequences, etc.

It is envisaged that a protein, glycoprotein, lipoprotein, or combination of proteins including cell extracts etc. may be used as starting point for the chemical stepwise synthesis of, e.g., sugars, vitamins, lipids, steroids, cytotoxic compounds or other chemical compounds.

The new products made possible through the invention are important new tools in biotechnology. Thus, one aspect made possible through the invention is a method of producing an antibody specific for a peptide such as a peptide hormone, a peptide growth factor, or a peptide with another defined biological effect, which comprises immunizing an animal with a coupled product according to the invention in which the molecule coupled to the proteinaceous substance is the peptide to which the specific antibody is to be raised, or an immunogenically effective part of the peptide, to make cells in the animal produce an antibody specific for said peptide, and obtaining such cells from the animal, or isolating the antibody from serum or plasma of the animal.

This method may further comprise:

fusing cells producing antibody with cells of a suitable cell line, and selecting and cloning the resulting hybridoma cells producing the antibody, or

immortalizing an unfused cell line producing antibody,

followed by growing the cells in a suitable medium to produce said antibodies,

and harvesting the antibody from the growth medium.

The animal immunized may be selected from the group consisting of rabbit, monkey, sheep, goat, mouse, rat, pig, horse, and guinea pig.

The cells producing the antibody are normally spleen or lymph cell.

Another interesting aspect of the invention is solid phase enzyme immunoassay or radioimmunoassay using as the solid phase coupled protein–peptide products according to the invention.

Coupled products of the invention are alse highly interesting as vaccines, in particular when the protein is a serum protein. Thus, one aspect of the invention is a method of immunising an animal, including a human being, e.g. against a disease caused by a microorganism or a virus, comprising parenterally administering a coupled product according to the invention in which the coupled group is an immunogenic peptide sequence of a protein, e.g. a protein of a microorganism or virus.

A further aspect of the invention is a method of purifying antibodies against a peptide, comprising applying a medium containing the antibodies to a suspension or solution of a coupled product according to the invention carrying a coupled peptide, to bind the peptide-specific antibodies to the peptide moiety of the coupled product, washing to remove unbound antibodies, cleaving the antibodies from the peptide moiety, and separating the antibodies from the medium, e.g. by dialysis.

In one aspect discussed above, the present invention relates to the use of the FS-coupled product (Functionality-Selectively coupled product) of the invention for immunizing purposes. The preparation of the FS-coupled product should be made so as to allow an optimal stimulation of the relevant parts of the immune system, i.e to present the immunogenic bound group for a period of time and in a form being optimal with respect to the recognization, the uptake or any other interaction or processing necessary for the stimulation.

The FS-coupled product of the invention may solely comprise T-cell epitopes or solely B-cell epitopes or a combination of these. Thus, the composition of the FS-coupled groups of the invention may be tailored to their intended use, e.g. their use as a vaccine component. B-cell epitopes are advantageous for most applica

tions as they are required for eliciting an antibody production. T-cell epitopes are extremely advantageous as they enhance and accelerate the immune response and the production of antibodies. Furthermore, the memory function of the immune system resides in the T-cells. By stimulating this part of the immune system, the antibody production is significant after approximately 5 days. If the memory function is not participating, e.g. in the non-immunized animal, or if the antigen used for immunization does not contain a T-cell epitope, the antibody production is significant after several weeks, consisting primarily of low avidity IgM antibodies and after several months consisting of IgG antibodies of higher avidity.

The FS-coupled product of the invention is contemplated to be useful as a prophylactic or therapeutic agent, e.g. a vaccine, or in a diagnostic kit and may be used in the manufacture thereof. This will be explained below.

The term "vaccine" is to be understood to comprise any preparation containing an immunologically effective part of a FS-coupled product suited for administration to living organisms for the prevention, amelioration or treatment of e.g. a bacterial or viral infection or hormonal disturbance. The term "immunization" is understood to comprise the process of evoking a specific immunologic response with the expectation that this will result in humoral, and/or secretory, and/or cell-mediated immunity to the infection, i.e. immunity is to be understood to comprise the ability of the individual to resist or overcome infection or to overcome infection "more easily" compared to individuals who have not been immunized or to tolerate the infection without being clinically affected or to block transmission. Thus, the immunization according to the present invention is a process of increasing resistance to the bacterial or viral infection. An overall aspect in the preparation of the vaccines of the invention is the physiological acceptability of the components and of the total composition of the vaccine. The final formulation of the vaccine should be a mixture of substances supporting and enhancing the immune response induced by the specific immunogenic component.

In another preferred embodiment of the invention, a multivalent vaccine is formulated, i.e. several immunologically effective components are incorporated into a single vaccine being effective in reducing infection, and/or transmission – all in all

inducing an effective protective immunity. The vaccine may comprise one or more selected specific molecules in order to provide the multivalent nature of the vaccine. Especially interesting additional molecules are immunologically active molecules obtained from pathogenic organisms which give rise to a vaccine being effective in reducing infection or providing immunity for one or more pathogenic organisms.

Routine methods for vaccine production involve risks of obtaining unwanted side effects, e.g. due to the vaccine containing unwanted (or even unidentified) contaminants. The methods of preparation of vaccines according to the present invention are designed to ensure that the identity and immunological effectiveness of the specific molecules are maintained and that no unwanted microbial contaminants are introduced. The final products are distributed under aseptic conditions into preferably sterile containers which are then sealed to exclude extraneous microorganisms.

The vaccine may further comprise an adjuvant in order to increase the immunogenicity of the vaccine preparation. The adjuvant may serve the purpose of enhancing the stimulatory properties of the polypeptide by stimulating the production of cytokines or lymphokines from the cells of the immune system in a non-specific way. The adjuvant may be selected from the group consisting of Freund's incomplete adjuvant, aluminium hydroxide, a saponin, a muramyl peptide, a lipopolysaccharide, a T-cell immunogen, interleukin-2, interferon-gamma, an oil, such as a vegetable oil, e.g. peanut oil, or a mineral oil, e.g. silicone oil, and B.C.G.

The bound group may advantageously remain coupled to the proteinaceus substance, which may be any carrier usually employed in the preparation of vaccines. The proteinaceus substance may be a e.g. a polypeptide such as a serum protein to which the selected immunologically interesting group or groups are covalently or non-covalently bound. The carrier should preferably be non-toxic and non-allergenic such as serum albumin. The bound group may be multivalently bound to the macromolecular carrier as this provides an increased immunogenicity of the vaccine preparation. In this regard, it may prove advantageous to couple several different groups to the carrier so as to obtain a vaccine comprising a

variety of different immunogenic determinants, i.e. a cocktail vaccine, which may be employed for the immunization of diseases caused by a variety of different organisms.

Various immunization schedules may be employed when using the vaccine of the invention: In some instances it may be appropriate to provide active immunization early in life. Furthermore, it may be desirable to employ repeated administrations, e.g. at regular or prolonged intervals, optionally – as far as injections are concerned – at various body sites, e.g. at the same time. Any immunization schedule which may be contemplated or shown to produce an appropriate immune response can be employed in accordance with the principles of the present invention.

The vaccine should be administered in a way which ensures an efficient stimulation of the immune system. This means that the vaccine should be brought into contact with the cells of the immune system for a sufficient period of time and in a form capable of functioning as an immunogen. Several ways are possible. Of these the most conventional are the parenteral ways, i.e., the subcutaneous, intradermal, intramuscular or the intravenous route.

Other more unusual ways of administering the vaccine are the nasal, the oral or the rectal routes. A combination of the two, first mentioned routes could be achieved by using an aerosol formulation of the vaccine to be administered via the respiratory tract. This formulation of a vaccine has been proposed for special purposes where a more conventional formulation would be insufficient due to: the need for vaccination in very remote areas, the logistic problems associated with transportation and storage of the vaccine, problems associated with infections being spread by multiple use of syringes and the need to vaccinate large populations.

The aerosol vaccine is in most cases administered via the nasal route. It is known that peptides can be transported intactly through the nasal mucosa to reach the blood. When transported further down the respiratory tract, the antigen is taken up by the macrophages functioning as scavengers and is in this way potentially presented to the immune system. Some of the material administered as an aerosol

may possibly reach the intestines and stimulate the immune system present in the intestines and this way stimulate the immune system of the body, or may be taken up by the intestinal mucosa in intact form and liberated to the blood stream where it will be presented for the immune system.

The vaccine may also be administered strictly via the nasal route. This way simplifies the administration and circumvents the problems associated with spreading of infectious diseases through multiple use of syringes.

Another aspect of the invention is a monoclonal or polyclonal antibody reactive with a bound group on the FS-coupled product, or an immunologically subsequence thereof, and a method for the preparation thereof. The term "antibody" refers to a substance which is produced by a mammal or a more precisely a cell of mammalian origin belonging to the immune system as a response to exposure to the FS-coupled products of the invention.

The antibodies of the present invention may be produced by a method which comprises administering to an animal the FS-coupled product of the invention thereby presenting the bound groups of the FS-coupled product in an immunogenic form so as to make cells producing antibodies reactive with said bound groups, and isolating the cells producing the antibodies or serum or plasma containing the antibodies from the animal. The methods of producing the antibodies of the present invention will be explained further below.

The antibody is preferably a monospecific antibody. The monospecific antibody may be prepared by injecting to a suitable animal a substantially pure preparation of the FS-coupled product of the invention followed by one or more booster injections at suitable intervals (e.g. one or two weeks to a month) up to four or five months before the first bleeding. The established immunization schedule is continued, and the animals are bled about one week after each booster immunization, and antibody is isolated from the serum in a suitable manner (cf. e.g. Harboe and Ingild, Scand. J. Immun. 2 (Suppl. 1), 1973, pp. 161-164.)

For purposes not requiring a high assay specificity, the antibody may be a polyclonal antibody. Polyclonal antibodies may be obtained, e.g. as described in Harboe and Ingild, see above. More specifically, when polyclonal antibodies are to be obtained, the FS-coupled product is, preferably after addition of a suitable adjuvant, such as Freund's incomplete or complete adjuvant such as a muramyl peptide, injected into an animal. When the immunogens are human FS-coupled products, the animals may be rabbits. The animals are bled regularly, for instance at weekly intervals, and the blood obtained is separated into an antibody containing serum fraction, and optionally said fraction is subjected to further conventional procedures for antibody purification, and/or procedures involving use of a purification procedure in which the FS-coupled product in question is used.

In another preferred embodiment, monoclonal antibodies are obtained. The monoclonal antibody may be raised against or directed substantially against an essential component of the bound group of the FS-coupled product, i.e. an epitope. The monoclonal antibody may be produced by conventional techniques (e.g. as described by Köhler and Milstein, Nature 256, 1975, p. 495) e.g. by use of a hybridoma cell line, or by clones or subclones thereof or by cells carrying genetic information from the hybridoma cell line coding for said monoclonal antibody. The monoclonal antibody may be produced by fusing cells producing the monoclonal antibody with cells of a suitable cell line, and selecting and cloning the resulting hybridoma cells producing said monoclonal antibody. Alternatively, the monoclonal antibody may be produced by immortalizing an unfused cell line producing said monoclonal antibody, subsequently growing the cells in a suitable medium to produce said antibody, and harvesting the monoclonal antibody from the growth medium.

The immunized animal used for the preparation of antibodies of the invention is preferably selected from the group consisting of rabbit, monkey, sheep, goat, mouse, rat, pig, horse and guinea pigs. The cells producing the antibodies of the invention may be spleen cells or lymph cells, e.g. peripheric lymphocytes.

When hybridoma cells are used in the production of antibodies of the invention, these may be grown in vitro or in a body cavity of an animal. The antibody-

producing cell is injected into an animal such as a mouse resulting in the formation of an ascites tumour which releases high concentrations of the antibody in the ascites of the animal. Although the animals will also produce normal antibodies, these will only amount to a minor percentage of the monoclonal antibodies which may be purified from ascites by standard purification procedures such as centrifugation, filtration, precipitation, chromatography or a combination thereof. A purification procedure using the FS-coupled product in question is especially preferred.

An example of a suitable manner in which the monoclonal antibody may be produced is as a result of fusing spleen cells from immunized mice (such as Balb/c mice) with myeloma cells using conventional techniques (e.g. as described by R. Dalchau, J. Kirkley, J.W. Fabre, "Monoclonal antibody to a human leukocyte—specific membrane glycoprotein probably homologous to the leukocyte-common (L-C) antigen of the rat", Eur. J. Immunol. 10, 1980, pp. 737-744). The antibodies resulting from the obtained fusions are screened by conventional techniques such as binding assays e.g. an ELISA assay employing the FS-coupled product in question.

The FS-coupled products of the invention are useful in e.g. a diagnostic agent in which the presence of antibodies against the bound group of the FS-product in question is to be determined. The diagnostic agent may be one which is suited for use in an agglutination assay in which the solid particles to which the FS-coupled product is coupled agglutinate in the presence of antibodies against the bound group of the FS-coupled product in the sample subjected to testing. In this type of testing, no labelling of the antibody is necessary. For most uses it is, however, preferred that the FS-coupled product is provided with a label for the detection of bound antibody. The substance used as label may be selected from any substance which is in itself detectable or which may be reacted with another substance to produce a detectable product. Thus, the label may be selected from radioactive isotopes, enzymes, chromophores, fluorescent or chemiluminescent substances, and complexing agents.

Examples of enzymes useful as labels are ß-galactosidase, urease, glucose oxidase, carbonic anhydrase, peroxidases (e.g. horseradish peroxidase), phospha

tases (e.g. alkaline or acid phosphatase), glucose-6-phosphate dehydrogenase and ribonuclease.

Enzymes are not in themselves detectable, but must be combined with a substrate to catalyze a reaction the end product of which is detectable. Thus, a substrate may be added to the reaction mixture resulting in a coloured, fluorescent or chemiluminescent product or in a colour change or in a change in the intensity of the colour, fluorescence or chemiluminescence. Examples of substrates which are useful in the present method as substrates for the enzymes mentioned above are H_2O_2, p-nitrophenylphosphate, lactose, urea, ß-D-glucose, CO_2, RNA, starch, or malate. The substrate may be combined with, e.g. a chromophore which is either a donor or acceptor.

Fluorescent substances which may be used as labels for the detection of the components as used according to the of invention may be 4-methylumbelliferyl-phosphate, 4-methylumbelliferyl-D-galactopyranoside, and 3-(p-hydroxyphenyl) propionic acid. These substances may be detected by means of a fluorescence spectrophotometer. Chemiluminescent substances which may be peroxidase/eosin/EDTA, isoluminol/-EDTA/H_2O_2 and a substrate therefor.

Chromophores may be o-phenylenediamine or similar compounds. These substances may be detected by means of a spectrophotometer.
Radioactive isotopes may be any detectable and in a laboratory acceptable isotope, e.g. ^{125}I, ^{131}I, ^{3}H, ^{35}P, ^{35}S or ^{14}C. The radioactivity may be measured in a ©-counter or a scintillation counter or by radioautography followed by densitometry.

Complexing agents may be Protein A, Protein G (which forms a complex with immunoglobulins), biotin (which forms a complex with avidin and streptavidin), and lectin (which forms a complex with carbohydrate determinants, e.g. receptors). In this case, the complex is not in itself directly detectable, necessitating labelling of the substance with which the complexing agent forms a complex. The marking may be performed with any of the labelling substances described above.

In an embodiment of the invention an antibody or a FS-coupled product of the invention may be coupled to a bridging compound coupled to a solid support. The bridging compound, which is designed to link the solid support and the antibody may be hydrazide, Protein A, glutaraldehyde, carbodiimide, or lysine.

The solid support employed is e.g. a polymer or it may be a matrix coated with a polymer. The matrix may be of any suitable solid material, e.g. glass, paper or plastic. The polymer may be a plastic, cellulose such as specially treated paper. nitrocellulose paper or cyanogenbromide-activated paper. Examples of suitable plastics are latex, a polystyrene, polyvinylchloride, polyurethane, polyacrylamide, polyvinylacetate and any suitable copolymer thereof. Examples of silicone polymers include siloxane.

The solid support may be in the form of a tray, a plate such as a mitrotiter plate, e.g. a thin layer or, preferably, strip, film, threads, solid particles such as beads, including Protein A-coated bacteria, or paper.

The FS-coupled product and the antibody raised against the bound group of the FS-coupled product according to the present invention may be used in an assay for the identification and/or quantification of said FS-coupled product or antibody reacting with at least part of the FS-coupled product present in a sample. Both a qualitative and a quantitative determination may be obtained according to the use of the present invention. The identification and/or quantification of said FS-coupled product or antibody reacting with at least part of the FS-coupled product may be performed for both a scientific, a clinical and an industrial purpose.

The antibody used in the present method is preferably in substantially pure form (purified according to suitable techniques or by the methods of the invention, see below) in order to improve the precision and/or accuracy of the assays of the invention.

The determination of antibodies reactive with the FS-coupled product of the invention and being present in a sample, e.g. as defined above, may be carried out by use of a method comprising contacting the sample with the FS-coupled

product of the invention and detecting the presence of bound antibody resulting from said contacting and correlating the result with a reference value.

When the FS-coupled product of the invention is to be employed in an assay for determining the presence of antibodies against the FS-coupled product in a sample, it may be in the form of a diagnostic reagent or a diagnostic agent. As will be apparent to a person skilled in the art several techniques may be applied in connection with such diagnostic reagents.

In a yet other aspect the present invention relates to administration of the various above-mentioned functionality-specifically coupled products to a mammal, preferably a human being, may be performed by any administration method which is suitable for administering proteins or peptides or antibodies. Typical administration routes are parenteral, oral, nasal, topical or rectal administration. In each case, the functionality-specifically coupled products to be administered should be formulated in a manner which will protect the functionality-specifically coupled products against degradation, in particular by enzymes. In many cases, the parenteral administration is the safest way of administering proteins and peptides. The parenteral administration route should be selected dependent on where the functionality-specifically coupled products is to be released, e.g. intravenously, intramuscularly or subcutaneously, etc. It is also important to consider the necessity of "packing" the functionality-specifically coupled products in a suitable manner in order to

1) obtain a sufficient therapeutic concentration level for a suitable time,
2) avoid first-pass metabolism,
3) avoid allergic and immunological reactions, and
4) avoid undesired side effects by
5) obtaining transport of the functionality-specifically coupled products to the site of action.

When the functionality-specifically coupled products is administered perorally, suitable measures should be taken to protect the functionality-specifically coupled products from enzymatic degradation in the gastrointestinal tract, e.g. by packing

the functionality-specifically coupled products in such a way that it will not be released from the formulation (i.e. the pharmaceutical composition) until it has reached the site where either the functionality-specifically coupled products is to exert its activity locally (i.e. in the gastrointestinal tract) or from where the absorption may take place (e.g. M-cells in the colon).

When rectal administration is performed, it is often desirable to use the so-called enhancers which are capable of making functionality-specifically coupled productss of the peptide type pass the rectal mucosa and thereby become absorbed.

Nasal administration is an administration form which is presently intensively investigated in order to provide absorption of substances of the peptide type from the nasal cavity. In principle, this may take place in two ways, firstly by using enhancers, and secondly by using the bioadhesion principle in which the functionality-specifically coupled products may be maintained for a long period of time at a suitable domain in the nose.

Topical administration may be performed by formulating the functionality-specifically coupled products in a salve, an ointment, a lotion, a creme, etc.

Formulations for parenteral use may be presented in unit dose forms, e.g. ampoules, or in multi-dose containers with an added suitable preservative. The composition may be in form of a solution, a suspension, an emulsion or a delivery device for implantation or may be presented as a dry powder to be reconstituted with water or another suitable solvent before use. Apart from the FS-product the compositions may comprise suitable pharmaceutically acceptable carriers and/or exipients. Furthermore the composition may, in addition, conveniently comprise suspending, stabilizing, pH-adjusting agents and/or dispersing agents.

Compositions for oral or rectal use may be formulated according to conventional pharmaceutical practice and may be in the form of tablets, capsules, pills, powders, ampoules, granulates, dragées, pastes, gels, suppositories or enemas; or liquid formulations such as suspensions (oily or aqueous), solutions, elixirs, emulsions or drenches and the like.

The auxiliary additives of the pharmaceutical compositions may be any conventional pharmaceutical additives and carriers:

Binding agents such as cellulose derivatives, starch, gelatin and polyvinylpyrrolidine; fillers such as sugar, mannitol, lactose, microcrystalline cellulose, potato starch, calcium phosphate; suitable absorption promotors; disintegrants such as potato starch, alginic acid; lubricants such as magnesium stearate, stearic acid, talc; emulsifying agents such as lecithin, sorbitan monooleate; wetting agents such as lecithin, polyoxyethylene esters; or buffering agents such as acetate, citrate, phosphate.

The solid compositions may by means of specially adapted coating techniques be provided with a coating adapted to protect the composition from unwanted chemical changes prior to the release of the active compound. The coating may be adapted to release the active compound in a predetermined pattern e.g. in order to achieve a controlled release formulation.

Pharmaceutical compositions for topical use such as compositions suitable for application to the skin according to the present invention are suitably creams, gels, ointments, lotions, liniments, suspensions, solutions, pastes, sticks, sprays, soaps, shampoos, powders, suppositories and enemas. The topical administration should be onto or close to the pathological changes in the body. The compositions may be any suitable medicated mass adapted for direct application or for introduction into the relevant orifice of the body, e.g. the rectal, urethral or vaginal orifice. The compositions may simply be applied directly onto the diseased part, e.g. the skin or mucosa. Other relevant formulation adaptations for application to the eye may e.g. be eye lotions, eye ointments, eye-drops or drug delivery systems adapted for administration to the eye such as compositions suitable for implantation administration. In certain cases it may be applied by means of special medical devices such as dressings including occlusive dressings, or alternatively plasters, pads, sponges, strips, or other forms of suitable flexible pieces of material.

Formulations for topical administration may comprise pharmaceutically acceptable carriers and/or exipients such as ointment bases (e.g. paraffin, polyethylene

glycols, Tween ®, Span ®, vegetable oils), suspending or emulsifying agents (e.g. lecithin, sorbitan monooleate, gelatin, methyl cellulose, gum acacia, sorbitan monooleate derivatives), gelforming agents (e.g. Carbopol, alginates, gelatin), preservatives (e.g. methyl or propyl p-hydroxybenzoates, benzalkonium chloride), antioxidants (e.g. tocopherol, ascorbic acid, butylated hydroxy anisol), humectants (e.g. glycerin, propylene glycol, urea), and perfumes and skin protective agents.

Formulations for topical administration to the eye may comprise pharmaceutically inert vehicles or be in form of a suitable carrier system. Pharmaceutically acceptable carriers and/or exipients for the preparation of eyedrops include for example buffering agents such as boric acid or borates, pH adjusting agents to obtain optimal stability or solubility of the actuve drug substance, agents to adjust the tonicity such as sodium chloride or borates, viscosity altering agents such as hydroxypropyl cellulose, methylcellulose, polyvinylpyrrolidone, polyvinyl alcohols or polyacrylamide, oily vehicles such as vehicles comprising arachis oil, castor and mineral oils. Eyedrops presented as emulsions or suspensions may furthermore comprise stabilizing, dispersing, wetting, emulsifying and/or suspending agents. Eye lotions and eye ointments may comprise pharmaceutically acceptable carriers and/or exipients such as used in an eyedrop composition or in other relevant topical composition, e.g. ointments, creams, lotions and the like.

In a further aspect of the invention, antibodies highly specific for the N-terminal part or the C-terminal part of the coupled peptide or amino acid are obtained which may be used for the determination of the C- or N-terminal amino acids in other peptides or proteins before and after proteolytic or chemical fragmentation and separation of the fragments.

The invention is further illustrated by the following non-limiting examples given below, in which the cheap and readily available protein bovine serum albumin (BSA) is employed as proteinaceous synthesis substrate.

Abbreviations used in the present specification and claims are as follows:

Boc	tert-butyloxycarbonyl
BOP	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BSA	bovine serum albumin
tBu	tert-butyl
DCM	dichloromethane
DEA	diethylamine
Dhbt-OH	3-hydroxy-3,4-dihydrobenzotriazin-4-one
DIPCDI	diisopropylcarbodiimide
DMAP	4-(dimethylamino)pyridine
DMCS	dimethyldichlorosilane
DMF	N,N-dimethylformamide
Fmoc	9-fluorenylmethyloxycarbonyl
HSA	human serum albumin
HMPA	4-(hydroxymethyl)phenoxyacetic acid
NHS	N-hydroxysuccinimide
PBS	phosphate-buffered saline
Pfp	pentafluorophenyl
PPD	purified protein derivative
TEA	triethylamine
TFA	trifluoroacetic acid
TNT	tetanus toxoid
TRIS	tris(hydroxymethyl)aminomethane

EXAMPLE 1

Silylation of glass equipment.

Procedures making use, according to the invention, of a proteinaceous substrate for solid-phase peptide synthesis may be carried out in equipment of the type used for conventional solid-phase peptide synthesis with synthetic polymer substrates. A preferred type of reaction vessel has the form of a separating funnel, in the lower part of the inside of which there is a sintered glass filter which separates the inside of the "funnel" into upper and lower compartments and upon

which a weighed amount of the appropriate proteinaceous substrate is placed. The upper end of the vessel is equipped with a screw stopper permitting the introduction of activated and protected amino acids. The lower end of the vessel is equipped with a two-way stopcock, e.g. of glass, having two tubing connections and facilitating the introduction of liquid reagents and solvents by firstly applying vacuum to the reaction vessel via one tubing connection of the stopcock, and then allowing the liquid medium in question to enter the evacuated vessel via the other tubing connection of the stopcock. Liquid media are removed from the reaction vessel via the tubing connection used for applying vacuum. During the peptide synthesis procedure the reaction vessel is positioned in a shaking apparatus. Before starting the synthesis, glass parts of the reaction vessel are silylated in the following manner (procedure based on that described by J.M.Stewart and J.D. Young Solid Phase Peptide Synthesis, p. 70, Pierce Chemical Comp. 2nd. ed., 1984):

The glass parts are immersed in a 10% solution of dimethyldichlorosilane (DMCS) in dry toluene for 20 min, followed by washing with toluene (3 times), immersion in dry methanol for 20 min They are then washed with methanol and finally with dry acetone. The glass parts are then dried at 120 °C overnight.

EXAMPLE 2

Blocking of protein side-chain carboxyl groups by diethylamidation.

BSA contains 59 lysine units, of which 29 have been reported to be exposed for conjugation to peptides (L. Aldwin and D. Nitekci Anal.Biochem. 164 (1987) 494). The molecular weight of the protein is approximately 70,000 dalton, corresponding to 0.43 mequiv. of exposed amino groups per gram of BSA.

BSA (0.194 g; Sigma, A-4503), DIPCDI [MW 126.2; 0.194 g x 0.43 mequiv./g x 126 g/equiv. x 10-fold excess = 0.105 g = 128 µL (d = 0.82 g/ml)] and DMF (2 ml) were placed in the reaction vessel. A ten-fold excess of DEA (89 µL; distilled over ninhydrin) in DMF (3 ml) was added and the mixture shaken for 30 min. The solvent was removed and the product was washed with DMF (5x3 ml) and then

with DCM (2x5 ml). The product (BSA diethylamide) was dried by applying vacuum to the reaction vessel. Weight 0.189 g.

EXAMPLE 3

Synthesis of H-Leu-Leu-Ala-Gly-Val-OH using BSA diethylamide as substrate.

BSA (0.297 g) was placed in the silylated reaction vessel and the carboxylic acid groups blocked as described in example 2. HMPA-Dhbt ester (MW 327.2) (0.297 x 0.43 x 327 x 3-fold excess = 0.126 g) in DMF (5 ml) was added and the mixture shaken for 2 hours.

The protein turned yellow due to deprotonation of Dhbt-OH by the unacylated amino groups with the formation of Dhbt-O^-. As the acylation reaction proceeded, the colour of the protein gradually faded to pale yellow. The solution was drained from the reaction vessel, and the solid product was washed with DMF (5x3 ml) and DCM (2x5 ml) and dried in vacuo for 30 min. Weight of HMPA-BSA diethylamide: 0.287 g.

The first Fmoc-protected amino acid (Fmoc-Val-OH, MW 339.4) was added as the anhydride in 10-fold excess: Fmoc-Val-OH (0.297 x 0.43 x 339 x 10-fold excess = 0.432 g) was dissolved in DCM (5 ml), cooled to 0°C, and 5 equiv. of DIPCDI (0.297 x 0.43 x 126 x 5-fold excess = 0.080 g = 98 µL) was added and the mixture left for 10 min with stirring. The solvent was removed in vacuo and the solid anhydride was immediately dissolved in DMF (5 ml). The resulting solution was added to the HMPA-BSA diethylamide in the reaction vessel. One equiv. of DMAP (MW 122.2) (0.297 x 0.43 x 122 x 1 = 15.6 mg) was then added (as the last reagent in order to avoid racemization of the anhydride) as acylation catalyst and the mixture shaken for 2 hours. A ten-fold excess of acetic anhydride (MW 102.1; d = 1.08 g/ml) (0.297 x 0.43 x 102 x 10-fold excess = 0.130 g = 121 µL) and TEA (MW 101.2; d = 0.726 g/ml) (0.297 x 0.43 x 101 x 10-fold excess = 0.129 g = 178 µL) was added as capping reagent to inactivate remaining free amino groups, and the mixture was shaken for 10 min. The solvent was drained

from the reaction vessel and the product washed with DMF (5 x 3 ml) and DCM (2 x 5 ml) and dried in vacuo. At this point the solid product obtained was colourless. Weight: 0.261 g.

Piperidine was used to effect removal of the Fmoc protecting group. Thus, a 20% solution of piperidine in DMF (5 ml) was added to Fmoc-Val-BSA (0.271 g) and the mixture shaken for 5 min. After draining, the process was repeated. The product was washed with DMF (5 x 3 ml) and DCM (2 x 5 ml) and the product dried in vacuo. Weight: 0.251 g.

The next amino acid was coupled as the Fmoc-Gly-Dhbt ester. Thus, a solution of Fmoc-Gly-Dhbt (MW 442.4) (0.297 x 0.43 x 442 x 3-fold excess = 0.149 g) in DMF (5 ml) was added and the mixture shaken for 2 hours. The initially strongly yellow coloured protein gradually turned pale yellow but did not become completely decolourized. The solvent was drained from the reaction vessel, and the product was washed with DMF (5 x 3 ml), DCM (2 x 5 ml) and dried in vacuo. Weight: 0.284 g. To effect removal of the Fmoc group the above deprotection scheme was employed.

The following amino acids in the sequence: Fmoc-Ala^{3}-Dhbt (MW 456.5; 0.297 x 0.43 x 456.5 x 3-fold excess = 0.174 g), Fmoc-Leu^{4}-Dhbt (MW 498.5; 0.297 x 0.43 x 498 x 3-fold excess = 0.191 g) and Fmoc-Leu^{5}-Dhbt (MW 498.5; 0.297 x 0.43 x 498 x 3-fold excess = 0.191 g) were coupled analogously. After each coupling and each Fmoc-deprotection step the product was weighed in order to observe the weight increase; in some cases this may be negligible because of loss of inclusion water from the BSA (concerning inclusion water in BSA, see, e.g., A.Shinohara, K.Nunomura and T.Fujita, J.Polymer Science: Polymer Symp. 69 (1981) 21; J.Wiley and Sons inc.), probably as the result of conformational changes occurring during the synthesis (Table 1).

The peptide was cleaved from the protein using 95% aqueuos TFA followed by work-up of the product. Cleavage and work-up were carried out according to either of the following two procedures:

A. Peptide-HMPA-BSA diethylamide (0.125 g) was dissolved in 95% TFA (2.5 ml) and left for 2 hours at room temperature in a closed flask. The resulting gelatinous product was dissolved in additional 95% TFA (5 ml) and the solvent then removed in vacuo on a rotary evaporatoer. Dry diethyl ether (20 ml) was added, and the mixture was left for 10 min at room temperature and the ether decanted from the solid product. This process was repeated twice. The product was dried with a slow stream of nitrogen for ca. 30 min. Weight of the crude product: 0.089 g. The product was dissolved in water (2 ml), transferred to a centrifree™ membrane (Amicon Division, W.R.Grace and Co., 24 Cherry Hill Drive, Danvers, Massachusetts 01923, USA) for separation of free peptide from protein and protein-bound microsolute and centrifuged (1000-2000 x g). The filtrate was collected and lyophilized to give the crude peptide. Weight: 0.020 g. This product was subjected to gel-filtration on Sephadex G10 (Pharmacia, Laboratory Separation Division, S-751 82 Uppsala, Sweden) in 1% acetic acid. The collected solutions were lyophilized to give 0.018 g of the pure peptide. The results of amino acid analysis of the peptide after gel-filtration are given in Table 2.

B. Peptide-HMPA-BSA diethylamide 0.125 g) was dissolved in 95% TFA (2.5 ml) and left for 2 hours at room temperature in a closed flask. The resulting gelatinous product was dissolved in additional 95% TFA (5 ml) and the solvent then removed in vacuo on a rotary evaporator. Dry diethyl ether (20 ml) was added, and the mixture was left for 10 min at room temperature and the ether decanted from the solid product. This process was repeated twice. The product was dried with a slow stream of nitrogen for ca. 30 min. Weight of the crude product: 0.082 g. The crude product was dissolved in water (2 ml) and dialyzed (exclusion limit ca. 30.000) (twice, 24 hours each time, 4°C) against water (50 ml). The two aqueous extracts gave on freeze drying 0.016 g and 0.001 g, respectively, of the pure peptide.

Table 1.

Weight increase (in grams) in each coupling step before and after removal of Fmoc protecting groups.

Amino acid	before	after
Gly^2	0.284	0.267
Ala^3	0.304	0.287
Leu^4	0.316	0.288
Leu^5	0.320	0.299

Table 2.

Amino acid analysis of H-Leu-Leu-Ala-Gly-Val-OH.

	Peptide from example 3A	Peptide from example 4
Val	1.00	1.00
Gly	1.13	1.12
Ala	1.05	1.06
Leu	1.99	1.96

EXAMPLE 4.

Synthesis of H-Leu-Leu-Ala-Gly-Val-OH on a "polyamide" resin.

The peptide was synthesized on a conventional "polyamide" resin [Macrosorb SPR 100 flow resin (Sterling Organics Ltd.)] for the purpose of comparison with the peptide synthesized on BSA in example 3. The resin (0.500 mg; substitution 0.1 mequiv./g) was activated with ethylenediamine, coupled with HMPA-Dhbt ester, and the first amino acid (Fmoc-Val-OH; MW 339.4; 0.5 x 0.1 x 339 x 10-fold excess = 0.170 g) was coupled as the anhydride using the method described in example 3. After deprotection with 20% piperidine in DMF, followed by subsequent

washings with DMF as described in example 3, the following amino acids were coupled as the Dhbt-esters using standard procedures (e.g. as in example 3): Fmoc-Gly-Dhbt, 73 mg; Fmoc-Ala-Dhbt, 75 mg; Fmoc-Leu-Dhbt, 82 mg, and Fmoc-Leu-Dbht, 82 mg. After completing the sequence, the resin was washed with ether and dried. The peptide was cleaved from the resin (0.250 g) by adding 5 ml of 95% aqueous TFA and leaving the mixture at room temperature for 2 hours. The solution was filtered from the resin and the TFA removed by evaporation with a slow stream of nitrogen. The product was treated with several small amounts of dry ether to give the crude peptide. The peptide was subjected to gel-filtration (Sephadex G10) in 1% acetic acid. The fractions containing the peptide were lyophilized to give 11 mg. The results of the amino acid analysis are given in Table 2.

EXAMPLE 5.

Synthesis of H-Phe-Leu-Glu-Glu-Val-OH using BSA diethylamide as substrate.

BSA (0.200 g) was placed in the silylated reaction vessel. The carboxylic acid groups were diethylamidated and the amino groups of were conjugated with HMPA-Dhbt ester as described in example 3 to give 0.202 g of HMPA-BSA diethylamide.

The first Fmoc-protected amino acid (Fmoc-Val-OH, MW 339.4) was added as the anhydride in 10-fold excess (0.200 x 0.43 x 339 x 10 = 0.292 g) using the method described in example 3.

The following amino acids in the sequence: Fmoc-Glu^{2}(OtBu)-Dhbt (MW 570.6; 0.200 x 0.43 x 570.6 x 3 = 0.147 g), Fmoc-Glu^{3}(OtBu)-Dhbt (0.147 g), Fmoc-Leu^{4}-Dhbt (MW 498.5; 0.200 x 0.43 x 498 x 3 = 0.129 g) and Fmoc-Phe-Dhbt (MW 532.6; 0.200 x 0.43 x 533 x 3 = 0.137 mg) were coupled using a three-fold excess and the Fmoc group removed after each coupling as described in example 3. After each of these steps the product was weighed (Table 3). Weight of H-Phe-Leu-Glu(OtBu)-Glu(OtBu)-Val-HMPA-BSA diethylamide: 0.320 g.

The peptide H-Phe-Leu-Glu-Glu-Val-OH was obtained by cleavage of the conjugate (0.125 g) with 95% TFA as described in example 3. The TFA treatment caused at the same time side-chain deprotection of glutamic acid. Weight of peptide/protein mixture: 0.093 g.

The peptide (from 0.125 g of the conjugate) may be purified as described in example 3A: Weight after "centrifree" purification and lyophilization: 0.027 g. Weight after gelfiltration and lyophilization: 0.025 g. Alternatively, the peptide (from 0.125 g) may be purified as described in example 3B: Weight after dialysis and lyophilization: 0.015 g.

Table 3.

Weight increase (in grams) in each coupling step before and after removal of Fmoc protecting groups.

Amino acid	before	after
Val[1]	0.247	0.220
Glu[2](tBu)	0.228	
	0.280*	0.256
Glu[3](tBu)	0.272	
	0.326*	0.286
Leu[4]	0.345	0.312
Phe[5]	0.351	0.320

*Double coupling

Table 4.

Amino acid analysis of H-Phe-Leu-Glu-Glu-Val-OH.

Val	0.99
Glu	1.89
Leu	1.00
Phe	0.97

EXAMPLE 6.

Synthesis of H-Leu-Leu-Ala-Gly-Val-BSA.

BSA (0.207 g) was placed in the silylated reaction vessel and Fmoc-Val-Dhbt (MW 484.5; 0.207 x 0.43 x 485 x 3-fold excess = 0.129 g) in DMF (5 ml) was added. The mixture was shaken for 2 hours. Remaining free amino groups on BSA were then blocked by addition of acetic anhydride (MW 102.1; d = 1.08 g/ml; 0.207 x 0.43 x 102 x 10-fold excess = 90.8 mg = 84 μL) and TEA (MW 101.2; d = 0.726 g/ml; 0.207 x 0.43 x 101 x 10 = 90 mg = 124 μL), the mixture being shaken for an additional 10 min. At this stage the initial yellow colour of the BSA, due to ionised Dhbt-OH, had vanished completely. The solvent was drained from the reaction vessel and the product washed with DMF (5 x 3 ml) and DCM (2 x 5 ml) and dried in vacuo. Weight: 0.226. To effect removal of the Fmoc group a 20% solution of piperidine in DMF (5 ml) was added and the mixture shaken for 5 min. After draining, the process was repeated. The product was washed with DMF (5 x 3 ml), and DCM (2 x 5 ml) and the product dried in vacuo. Weight: 0.207 g.

The following amino acids in the sequence: Fmoc-Gly^2-Dhbt (MW 442.4; 0.207 x 0.43 x 442 x 3 = 0.118 g), Fmoc-Ala^3-Dhbt (MW 456.5; 0.207 x 0.43 x 456.5 x 3 = 0.122 g), Fmoc-Leu^4-Dhbt (MW 498.5; 0.207 x 0.43 x 498 x 3 = 0.133 g) and Fmoc-Leu^5-Dhbt (MW 498.5; 0.207 x 0.43 x 498x3 = 0.133 g) were coupled analogously, except that blocking with acetic anhydride and DEA was omitted. Table 5 gives the weight of the product before and after deprotection in each of these steps. Coupling of Gly^2 and Leu^4 was repeated because of an unsatisfactory weight increase following one coupling procedure for these amino acids.

A total weight increase of 0.049 g was observed, corresponding to 1.08×10^{-4} mmol peptide (MW 454.6 for H-Leu-Leu-Ala-Gly-Val). In comparison with the starting amount of BSA (0.207 = ca. 2.96×10^{-6} mmol; MW ca. 70,000) this indicates formation of ca. 36 peptide chains conjugated by amide bonds to BSA. This is a minimum value, since some material may have been lost during washing procedures; furthermore, loss of crystal water present in the starting BSA is not accounted for.

The product was further purified in the following manner. H-Leu-Leu-Ala-Gly-Val-BSA (0.125 g) was transferred to a centrifuge tube and dissolved in DCM/TFA (1:1 v/v) (4 ml) at 0°C. After 5 min, dry diethyl ether (4 ml) was added, causing precipitation of the conjugate. The solvent was removed after centrifugation of the product, which was further washed three times with ether (3 x 4 ml) followed by drying in a stream of nitrogen. Weight: 0.116 g.

Table 5.

Weight increase (in grams) in each coupling step before and after removal of Fmoc protecting groups.

Amino acid	before	after
Gly^2	0.216	
	0.245*	0.222
Ala^3	0.261	0.231
Leu^4	0.255	
	0.271*	0.244
Leu^5	0.287	0.256

* Double coupling.

EXAMPLE 7.

Synthesis of H-Ser-Met-Asp-Thr-Ser-Lys-Glu-BSA diethylamide

BSA (0.196 g) was placed in the silylated reaction vessel and the carboxylic acid groups diethylamidated as described in example 2. Coupling of the first amino acid as Fmoc-protected Dhbt ester, blocking of remaining amino groups with acetic anhydride and coupling of the subsequent amino acids in the sequence in the form of Fmoc-protected Dhbt esters was performed as described in Example 6. The amino acid derivatives were used in three-fold excess in the following amounts: Fmoc-Glu^1(tBu)-Dbht (MW 570.6; 0.196 x 0.43 x 571 x 3 = 0.144 g); Fmoc-Lys^2(Boc)-Dhbt (MW 613.7; 0.155 g); Fmoc-Ser^3(tBu)-Dhbt (MW 528.6; 0.134 g);

Fmoc-Thr[4](tBu)-Dhbt (MW 542.6; 0.137 g); Fmoc-Asp[5](tBu)-Dhbt (MW 556.6; 0.141 g); Fmoc-Met[6]-Dhbt (MW 516.6; 0.131 g) and Fmoc-Ser[7](tBu)-Dhbt (MW 528.6; 0.134 g). Weight of H-Ser(tBu)-Met-Asp(tBu)-Thr(tBu)-Ser(tBu)-Lys(-Boc)-Glu(tBu)-BSA diethylamide: 0.268 g.

To effect side chain deprotection, H-Ser(tBu)-Met-Asp(tBu)-Thr(tBu)-Ser(tBu)-Lys(Boc)-Glu(tBu)-BSA diethylamide (0.109 g) was transferred to a centrifuge tube and dissolved in DCM/TFA (1:1 v/v) (4 ml) at 0°C. After 5 min, dry diethyl ether (4 ml) was added, causing precipitation of the conjugate. The solvent was removed after centrifugation of the product, which was further washed three times with ether (3 x 4 ml) followed by drying in a stream of nitrogen. The amino acid analysis of this conjugate is given in Table 6, together with the resultant amino acid composition of the peptide chain. The results indicate an average of 35 synthesized peptide chains per BSA molecule.

Sequence analysis results for the conjugate are shown in Table 7.

Table 6. Amino acid analysis of H-Ser-Met-Asp-Thr-Ser-Lys-Glu-BSA diethylamide and of BSA. Leu is used as standard value.

	BSA	Sequence derived composition	Conjugate	Composition	-BSA	Rel. amount
Amino acid						
Asp	897	53	1449	88.4	35.4	0.87
Thr	530	28	1109	67.7	39.7	0.97
Ser	418	24	1652	100.7	76.7	1.88
Glu	1352	81	1907	116.3	35.3	0.87
Pro	462	24	450			
Gly	272	12	282			
Ala	752	62	744			
Cys	377	35	394			
Val	537	43	552			
Met	57	6	767	46.8	40.8	1.00
Ile	194	9	213			
Leu	1000	61	1000	61.0	0	
Tyr	299	18	302			
Phe	432	31	436			
His	276	16	280			
Lys	961	59	1586	96.7	37.7	0.92
Arg	380	24	369			

Table 7. Sequence analysis of conjugate.

Step	Amino acid	pmol
1	Ser	374
2	Met	419
3	Asp	344
4	Thr	401
5	Ser	305
6	Lys	115
7	Glu	152

EXAMPLE 8

Raising of polyclonal antibodies against H-Ser-Met-Asp-Thr-Ser-Lys-Glu-BSA-diethylamide.

An immunization mixture was prepared which had the following composition: 1 ml of a 1 mg/ml suspension of H-Ser-Met-Asp-Thr-Ser-Lys-Glu-BSA (prepared as in example 7), 12.8 ml of 0.9% (w/v) NaCl solution (Merck, 6404), 2.2 ml of $Al(OH)_3$ solution (Alhydrogel, 6.9 mg/ml, Statens Seruminstitut, Copenhagen) and 150 µl of 0.01% merthiolate solution (Sigma, T-5125).

Three rabbits (Hvidsten, Statens Seruminstitut, Copenhagen) were immunized with the immunization mixture according to the following scheme: 1 ml immunization mixture was injected subcutaneously 5 times at intervals of 2 weeks, 1 ml of Freund's complete adjuvant (Statens Seruminstitut, Copenhagen) being mixed with the immunization mixture prior to the injection the first time, whereas 1 ml of Freund's incomplete adjuvant (Statens Seruminstitut, Copenhagen) was used in the same manner for the remaining 4 immunizations.

During the immunization period, the rabbits were bled the first time on day 10 after the second immunization, and subsequently 4 times at 2-weekly intervals. Bleeding was initiated by making a small transverse cut in a marginal ear vein. 40-50 ml of blood per bleeding were collected from each animal. The collected blood was left to coagulate at room temperature for 2 hours, after which the clot was loosened by a wooden spatula. The resulting coagulated blood was kept overnight at 4°C and centrifuged at 2,000 x g for 20 minutes. The blood serum was removed and stored until use at -20°C.

Testing for the presence of anti-H-Ser-Met-Asp-Thr-Ser-Lys-Glu antibodies in sera from immunized Balb/c mice by an ELISA technique:

Microtitre plates (Nunc, Roskilde, Denmark, 439454) were coated with 100 µl of H-Ser-Met-Asp-Thr-Ser-Lys-Glu-BSA in 50% TFA (0.2 mg/ml) by incubation overnight at room temperature. To each well was then added 50 µl of 10 M NaOH and 100 µl of 3 M TRIS.HCl (pH 8.8) and the plate was again incubated overnight at room temperature. The wells were then rinsed with distilled water and incubated with 0.1 M phosphate buffered saline (PBS) (pH 7.2) containing 1 mg BSA/ml and 0.1% Tween 20 (Merck) for 1 hour.

The thus prepared plates were next incubated with 100 µl/well of sera from the immunized rabbits or control serum diluted in PBS supplemented with 0.1% (w/v) bovine serum albumin (BSA, Sigma, A-4503) overnight at room temperature, using the following dilutions: 1:200, 1:400, 1:800, 1:1600, 1:3200 and 1:6400. After the incubation, plates were rinsed for 3 x 5 minutes with PBS containing 0.1% BSA, followed by incubation overnight at room temperature with peroxidase-labelled swine anti-rabbit antibodies (Dakopatts, Glostrup, Denmark) diluted 1:1000 in PBS containing 0.1% BSA, 100 µl/well, after which incubation the plates were rinsed for 3 x 5 minutes in PBS.

The presence of bound rabbit anti-H-Ser-Met-Asp-Thr-Ser-Lys-Glu antibodies as indicated by the amount of the peroxidase-labelled swine anti-rabbit antibodies optionally being bound to the rabbit anti-peptide antibodies was then tested for by adding to each well of the rinsed microtitre plates 100 µl of a staining buffer (7.3 g

citric acid, H_2O; 11.86 g Na_2HPO_4, H_2O; distilled water ad 1,000 ml) containing 0.04% (v/v) hydrogen peroxide (Perhydrol 30%) as the substrate and 0.4% (w/v) o-phenylenediamine dihydrochloride (Kem-En-Tec, Copenhagen, 3590) as the hydrogen donor (visualizing reagent).

The reaction was stopped by adding 125 µl of 1 M H_2SO_4/well.
The developed colour reaction was read as absorbance values using an InterMed, NJ2000 ELISA-reader operating at a wavelength of 490 nm. Figure 1 shows a plot of the absorbance at 490 nm (A_{490}) as a function of dilution.

The results clearly demonstrate that antibodies directed specifically against the H-Ser-Met-Asp-Thr-Ser-Lys-Glu sequence have successfully been raised using the peptide synthesised directly on, and conjugated to, the readily available protein substrate bovine serum albumin.

Claims

1. A method for the stepwise, controlled synthesis of chemical species, particularly peptides, the method comprising

(1) taking a proteinaceous substance, the proteinaceous substance conforming to at least one of the following criteria:

A. it is a polypeptide, oligopeptide or protein, as defined by the material comprising:

preferably at least 10 amino acid moieties, of which preferably at least two are different,

preferably at least 20 amino acid moieties, of which preferably at least two are different,

in particular at least 30 amino acid moieties, of which preferably at least two are different,

preferably at least 40 amino acid moieties, of which preferably at least two are different,

especially at least 50 amino acid moieties, at least two of which are different,

B. It comprises, as a significant part of its molecular structure, a polypeptide, oligopeptide, or protein as defined under A, the substance being, e.g., a glycoprotein, a lipoprotein, a protein conjugated with nucleic acids, or a substance comprising mixtures thereof, e.g. a bacterial cell, a bacterial cell wall fragment, or a cell extract,

(2) subjecting the proteinaceous substance to a chemical reaction, in a reaction medium, with a reactant selected from:

(i) an amino acid which is activated at the C-terminal carboxy group and at least N-terminal protected and in which any side-chain functionaliti es which can give rise to undesired reactions optionally are protected,

(ii) a peptide which is activated at the C-terminal carboxy group and at least N-terminal protected and in which any side-chain functiona lities which can give rise to undesired reactions optionally are protected,

(iii) any other molecule which is reactive towards available functionalities on the proteinaceous substance so as to form a bond between the pro teinaceous substance and the molecule, the molecule being, e.g, an amino sugar, a carboxylated sugar, an activated acid, an amine, an aldehyde or a ketone,

(3) removing excess reactant and reaction medium from the reaction product by filtration through a filter which allows passage of the reactant and reaction media, but substantially retains the reaction product,

(4) washing the reaction product with a washing medium, and removing the washing medium from the washed reaction product by filtration through a filter which allows passage of the washing medium, but substantially retains the reaction product, and

(5) repeating the cycle comprising steps as defined under (2)-(4) until the desired chemical species has been obtained, this repetition of steps as defined under (2)-(4) being optional when the coupling reactant is an amino acid or a peptide as defined under (2),

the reactant in the first step optionally being selected from:

1) reactants which, when coupled to the proteinaceous substance, provide a linker group useful as reaction target for a reactant selected from those defined under (2) (i), (2) (ii) and (2) (iii), the linker group being selected from

(a) groups which are capable of being cleaved under conditions which substantially do not deteriorate the reaction product formed on the linker group as a result of the treatment with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and

(b) groups which are adapted to provide a spacing (molecular distance) between the proteinaceous substance and the reaction product formed on the linker group as a result of the treatment with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and/or special physicochemical properties such as an increase or decrease of the solubility, in aqueous and/or organic solvents, of the proteinaceous substance carrying the linker group and the reaction product formed on the linker group as a result of the treatment with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and

2) reactants which permanently or reversibly blocking selected functional groups, in particular carboxylic acid groups, in the proteinaceous substance, such as primary or secondary amines, e.g. dialkylamines, e.g. diethylamine, and lower alkanolamines such as 2-ethanolamine, optionally combined with an activating reagent such as diisopropylcarbodiimide, dicyclohexylcarbodiimide or BOP reagent.

2. A method as claimed in claim 1, wherein the protein part of the proteinaceous substance is selected from naturally occurring proteins and modified naturally occurring proteins, such as:

egg white, albumins, in particular serum albumins (SA), including human serum albumin (HSA) and bovine serum albumin (BSA), parvoalbumin, α-lactalbumin,

ovalbumin, gelatin, lactoferrin, β-lactoglobulin, histones, keratins, keyhole limpet hemocyanin (KLH), tetanous toxoid (TNT), cholera toxin, hirudin, purified protein derivative (PPD), prolamins, hemocyanin, globins, hemoglobins, including methemoglobin, orosomucoid, transferrin, ferritin, apoferritin, ferritoxin, ceruloplasmin, haptoglobin, fibrinogen, fibrin, blood coagulation factors, Cohn blood fractions, agglutinins, plasmin, avidin, globulins, including α-globulins, e.g., α_1-globulin and α_2-globulin, such as α_2-macroglobulin, β-globulin, β_2-microglobulin, Θ-globulin, thyroglobulin, Cold insoluble globulin, lipoproteins, including α_1-lipoprotein, α_2-lipoprotein, β_1-lipoprotein and apolipoprotein, glycoproteins, including α_1-glycoprotein, enzymes, including lactic dehydrogenase, phosphorylase A and B, lysozyme, pepsin, carbonic anhydrase, urease, trypsinogen, trypsin, trypsin inhibitor, chymotrypsin, elastase, ribonuclease, amylases, including α-amylase and β-amylase, cholinesterase, peroxidases, catalases, alcohol dehydrogenase, cytochrome C, cytochrome C reduktase, reticulocyte lysate, fetuin, asialofetuin, C-reactive protein, α-foetoprotein, actin, elastin, myosin, tropomyosin, troponin, mitellogenin, myoglobin, collagen, protamine, lectin, including Con A, protein A, casein, glutens, zeins, yeast cell protein extract, bacterial cell protein extract, plant cell protein extract, animal cell protein extract and single cell protein,

or constitutes a part or fragment of any of the above-mentioned proteins or membranes.

3. A method according to claim 1 or 2, in which the coupling reactant is an amino acid as defined under (2) (i) or a peptide as defined under (2) (ii) in claim 1, and the cycle comprising steps (2)-(4) is performed two or more times with either such an amino acid or such a peptide in each step, the amino acid being the same or different in each step, and the peptide being the same or different in each step, to build up peptide chains on amino groups on the proteinaceous substance, the following steps being performed after each respective step (4):

(4a) deprotection of the N-terminal of the last-coupled amino acid or peptide prior to each new coupling reaction, the deprotection being performed by treatment with a deprotecting agent adapted to cleave off the protection group in question,

(4b) removing excess deprotecting agent and deprotecting media from the deprotected reaction product by filtration through a filter which allows passage of the deprotecting agent and deprotecting media, but substantially retains the deprotected reaction product, and

(4c) washing the deprotected reaction product with a washing medium, and removing the washing medium from the washed deprotected reaction product by filtration through a filter which allows passage of the washing medium, but substantially retains the deprotected reaction product,

optionally with final removal of any side-chain deprotecting groups present by

(4d) treatment with a side-chain deprotecting agent and removal of excess side-chain deprotecting agent and side-chain deprotecting media from the side-chain deprotected reaction product by filtration through a filter which allows passage of the side-chain deprotecting agent and side-chain deprotecting media, but substantially retains the side-chain deprotected reaction product, and

(4e) washing the side-chain deprotected reaction product with a washing medium, and removing the washing medium from the washed, side-chain deprotected reaction product by filtration through a filter which allows passage of the washing medium, but substantially retains the side-chain deprotected reaction product.

4. A method according to claim 3, in which amino groups of the proteinaceous substance are first reacted with a linker reactant which, when coupled to the proteinaceous substance, provides a linker group useful as reaction target for an amino acid as defined under (2) (i) or a peptide as defined under (2) (ii) in claim 1, the linker group being a group which is capable of being cleaved under conditions which substantially do not deteriorate the peptide formed on the linker group, the method optionally further comprising:

cleaving the linker group by providing conditions under which the group is cleaved, subsequently separating the peptide reaction product, formed on the linker group, from the proteinaceous substance by filtration through a filter which allows passage of the peptide product and the reaction media, but substantially retains the proteinaceous substance, and recovering the peptide product from the filtrate.

5. A method according to any of the preceding claims, wherein the proteinaceous substance is subjected to a treatment or treatments converting functional groups other than amino groups to linker groups useful as reaction target for a reactant selected from those defined under (2) (i), (2) (ii) and (2) (iii), the linker groups being selected from:

(a) groups which are capable of being cleaved under conditions which substantially do not deteriorate the reaction product formed on the linker group as a result of the treatment with a coupling reactant or coupling reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and

(b) groups which are adapted to provide a spacing (molecular distance) between the proteinaceous substance and the reaction product formed on the linker group as a result of the treatment with a coupling reactant or coupling reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii), and/or special physicochemical properties such as an increase or decrease of the solubility, in aqueous and/or organic solvents, of the proteinaceous substance carrying the linker group and the reaction product formed on the linker group as a result of the treatment with a coupling reactant or coupling reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii).

6. A method according to claim 5, wherein the groups converted are

carboxylic acid groups, which are converted into an amino-terminated group by treatment with a bifunctional compound, such as a diamine, e.g. ethylenediamine, capable of forming an amide bond with the carboxylic acid group and providing a free amino target group,

hydroxy groups which, e.g., are reacted with an α-haloacid to form a carboxy-terminated group which is subsequently converted into an amino-terminated group by treatment with a bifunctional compound, such as a diamine, e.g. ethylenediamine, capable of forming an amide bond with the carboxylic acid group and providing a free amino target group,

disulfide bonds, which are first reduced to SH groups and then reacted with an α-haloacid to form a carboxy-terminated group which is subsequently converted into an amino-terminated group by treatment with a bifunctional compound, such as a diamine, e.g. ethylenediamine, capable of forming an amide bond with the carboxylic acid group and providing a free amino target group.

7. A method according to claim 5 or 6, wherein one or several cycles as defined in claim 1 is/are performed on a species of functional group on the proteinaceous substance, such as amino, the reaction product, such as a peptide, from the cycle or the last of the cycles being left in at least N-terminal protected form, preferably in fully protected form including both side chain protection and N-terminal protection, after which the treatment or treatments according to claim 5 or 6 of another species of functional group is/are performed to convert the species into a target group, and the target group is subjected to reaction with one or more reactants selected from those defined under (2) (i), (2) (ii) and (2) (iii) in claim 1 throughout one or several cycles as described in claim 1.

8. A coupled product comprising a proteinaceous substance conforming to at least one of the following criteria:

A. it is a polypeptide, oligopeptide or protein, as defined by the material comprising:

preferably at least 10 amino acid moieties, of which preferably at least two are different,

preferably at least 20 amino acid moieties, of which preperably at least two are different,

in particular at least 30 amino acid moieties, of which preferably at least two are different,

preferably at least 40 amino acid moieties, of which preferably at least two are different,

especially at least 50 amino acid moieties, at least two of which are different,

B. It comprises, as a significant part of its molecular structure, a polypeptide, oligopeptide, or protein as defined under A, the substance being, e.g., a glycoprotein, a lipoprotein, a protein conjugated with nucleic acids, or a substance comprising mixtures thereof, e.g. a bacterial cell, a bacterial cell wall fragment, or a cell extract,

the substance carrying a group or a multitude of groups coupled thereto via a bond formed with a native or modified (e.g. via the introduction of a linker) functional group on the polypeptide, oligopeptide or protein part of the proteinaceous substance, the group or groups comprising a species selected from:

amino acid groups which are protected with a protecting group other than unsubstituted amide and other than lower alkyl (C_1-C_4) ester,

peptide groups, and

groups which can be prepared by reaction between native or modified (e.g. via the introduction of a linker) functional groups on the polypeptide, oligopeptide or protein part of the proteinaceous substance and molecules other than amino acid or peptide molecules which are reactive towards such functional groups to form a bond, in particular an amide bond or an ester bond, between the proteinaceous substance and the molecule in question, the molecule being, e.g, an amino sugar, a carboxylated sugar, an activated acid, an amine, an aldehyde or a ketone.

9. A coupled product according to claim 8, wherein the multitude of coupled groups is a number of groups less than or equal to the number of the relevant functional groups on the polypeptide, oligopeptide or protein part of the proteinaceous substance.

10. A coupled product according to claim 8 carrying a multitude of groups coupled thereto, the groups comprising at least two different species selected from:

amino acid groups which are protected with a protection group other than unsubstituted amide and other than lower alkyl (C_1–C_4) ester,

peptide groups, and

groups which can be prepared by reaction between native or modified (e.g. via the introduction of a linker) functional groups on the polypeptide, oligopeptide or protein part of the proteinaceous substance and molecules other than amino acid or peptide molecules which are reactive towards such functional groups to form a bond, in particular an amide bond or an ester bond, between the proteinaceous substance and the molecule in question, the molecule being, e.g. an amino sugar, a carboxylated sugar, an activated acid, an amine, an aldehyde or a ketone.

11. A coupled product according to claim 10, wherein the multitude of coupled groups is a number of groups less than or equal to the number of the relevant functional groups on the polypeptide, oligopeptide or protein part of the proteinaceous substance.

12. A coupled product according to claim 8 or 10, in which the protein part of the proteinaceous substance is selected from naturally occurring proteins and modified naturally occurring proteins, such as:

egg white, albumins, in particular serum albumins (SA), including human serum albumin (HSA) and bovine serum albumin (BSA), parvoalbumin, α-lactalbumin, ovalbumin, gelatin, lactoferrin, β-lactoglobulin, histones, keratins, keyhole limpet hemocyanin (KLH), tetanous toxoid (TNT), cholera toxin, hirudin, purified protein

derivative (PPD), prolamins, hemocyanin, globins, hemoglobins, including methemoglobin, orosomucoid, transferrin, ferritin, apoferritin, ferritoxin, ceruloplasmin, haptoglobin, fibrinogen, fibrin, blood coagulation factors, Cohn blood fractions, agglutinins, plasmin, avidin, globulins, including α-globulins, e.g., α_1-globulin and α_2-globulin, such as α_2-macroglobulin, β-globulin, β_2-microglobulin, ⊖-globulin, thyroglobulin, Cold insoluble globulin, lipoproteins, including α_1-lipoprotein, α_2-lipoprotein, β_1-lipoprotein and apolipoprotein, glycoproteins, including α_1-glycoprotein, enzymes, including lactic dehydrogenase, phosphorylase A and B, lysozyme, pepsin, carbonic anhydrase, urease, trypsinogen, trypsin, trypsin inhibitor, chymotrypsin, elastase, ribonuclease, amylases, including α-amylase and β-amylase, cholinesterase, peroxidases, catalases, alcohol dehydrogenase, cytochrome C, cytochrome C reduktase, reticulocyte lysate, fetuin, asialofetuin, C-reactive protein, α-foetoprotein, actin, elastin, myosin, tropomyosin, troponin, mitellogenin, myoglobin, collagen, protamine, lectin, including Con A, protein A, casein, glutens, zeins, yeast cell protein extract, bacterial cell protein extract, plant cell protein extract, animal cell protein extract and single cell protein,

or constitutes a part or fragment of any of the above-mentioned proteins.

13. A coupled product according to any of claims 8-12 wherein the coupled group or groups is/are a peptide group or peptide groups selected from one or several peptide species.

14. A coupled product according to claim 13, in which the peptide group or groups is/are selected from the group consisting of peptide hormones, and precursors, fragments, derivatives, antagonists and agonists thereof, the peptide hormones being, in particular, peptide hormones selected from the group consisting of

β-endorphin,
β-lipotropin,
⊖-endorphin,
gastrin releasing peptide,
growth hormone releasing factor,
neurotensin,

α-melanocyte stimulating hormone,
adrenocorticotropic hormone,
vasopressin,
angiotensin,
substance P,
dynorphin,
bradykinin,
luteinising hormone releasing hormone,
parathyroid hormone,
atrial natriuretic factor,
atriopeptin,
calcitonin,
calcitonin gene related peptide,
dermorphin,
kyotorpin,
auriculin,
ß-casomorphin,
tachykinin,
bombesin,
caerulein,
chorionic gonadotropin,
cholecystokinin,
casomorphin,
corticotropin,
corticotropin releasing factor,
corticotropin inhibiting peptide,
somatostatin,
eukephalin,
gastric inhibitory peptide,
gastrin,
gastrin related peptide,
thyrotropin releasing hormone,
glucacon,
growth hormone,

growth hormone releasing factor,
thyroid stimulating hormone,
prolactin,
neurokinin A and B,
neuromedin B, C, K, L, and U,
neuropeptide Y,
neurotensin,
oxytocin,
pancreastatin,
vasoactive intestinal peptide,
secretin,
galanin,
pancreatic polypeptide,
thymosin and
leucokinin.

15. A coupled product according to claim 13, in which the peptide group or groups is/are selected from the group consisting of peptide growth factors, and precursors, fragments, derivatives, antagonists and agonists thereof, the peptide growth factors being, in particular, peptide growth factors selected from the group consisting of

fibroblast growth factor,
transforming growth factor α,
transforming growth factor β I and II,
insulin,
cachetin,
epidermal growth factor,
bursin,
insulin-like growth factor I,
insulin-like growth factor II,
inhibin,
liver cell growth factor (Gly His Lys),
nerve growth factor,

platelet derived growth factor,
tuftsin, and
interleukins.

16. A coupled product according to claim 13, in which the peptide group or groups is/are selected from the group consisting of synthetic fragments of proteins with defined biological effect, and derivatives, fragments and analogues thereof, the proteins being, in particular, proteins selected from the group consisting of

fibronectin cell attachment sequence (Gly Asp Arg)
fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
leucine zipper sequence of, e.g., c-fos or c-jun,
helix-turn helix DNA binding sequence,
a protease inhibitor,
a kinase inhibitor,
a muramyl peptide,
zinc finger sequence,
B or T cell activating sequence,
nuclear translocation signal such as Lys Lys Lys Arg Lys,
memrane translocation signal and
glycosylation sequence (Asn X Ser/Thr),
homeobox sequences,

and any combination of these sequences with each other or with hormones, growth factors or derivatives thereof cited in claims 14 or 15.

17. A coupled product according to any of claims 13-16 in which the proteinaceous substance moiety is an albumin.

18. A coupled product according to claim 17, in which the albumin is human serum albumin.

19. A coupled product according to claim 17, in which the albumin is bovine serum albumin.

20. A coupled product according to claim 13 or claim 14, in which the protein is purified protein derivative (PPD).

21. A coupled product according to claim 13 or claim 14, in which the protein is tetanus toxoid (TNT).

22. A coupled product according to claim 17, the coupled product being human serum albumin (HSA) coupled to a hormone, and in particular being selected from the group consisting of

HSA-β-endorphin,
HSA-β-lipotropin,
HSA-Θ-endorphin,
HSA-gastrin releasing peptide,
HSA-growth hormone releasing factor,
HSA-neurotensin,
HSA-α-melanocyte stimulating hormone,
HSA-adrenocorticotropic hormone,
HSA-vasopressin,
HSA-angiotensin,
HSA-substance P,
HSA-dynorphin,
HSA-bradykinin,
HSA-luteinising hormone releasing hormone,
HSA-parathyroid hormone,
HSA-atrial natriuretic factor,
HSA-atriopeptin,
HSA-calcitonin,
HSA-calcitonin gene related peptide,
HSA-dermorphin,
HSA-kyotorpin,

HSA-auriculin,
HSA-β-casomorphin,
HSA-tachykinin,
HSA-bombesin,
HSA-caerulein,
HSA-chorionic gonadotropin,
HSA-cholecystokinin,
HSA-casomorphin,
HSA-corticotropin,
HSA-corticotropin releasing factor,
HSA-corticotropin inhibiting peptide,
HSA-somatostatin,
HSA-eukephalin,
HSA-gastric inhibitory peptide,
HSA-gastrin,
HSA-gastrin related peptide,
HSA-thyrotropin releasing hormone,
HSA-glucacon,
HSA-growth hormone,
HSA-growth hormone releasing factor,
HSA-thyroid stimulating hormone,
HSA-prolactin,
HSA-neurokinin A and B,
HSA-neuromedin B, C, K, L, and U,
HSA-neuropeptide Y,
HSA-neurotensin,
HSA-oxytocin,
HSA-pancreastatin,
HSA-vasoactive intestinal peptide,
HSA-secretin,
HSA-galanin,
HSA-pancreatic polypeptide,
HSA-thymosin and
HSA-leucokinin,

the hyphen designating a coupling amide bond between a functional group on HSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the HSA molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the HSA molecule,

and the corresponding coupled products where the peptide species coupled to the HSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone.

23. A coupled product according to claim 17, the coupled product being human serum albumin (HSA) coupled to a peptide growth factor, and in particular being selected from the group consisting of:

HSA-fibroblast growth factor,
HSA-transforming growth factor α,
HSA-transforming growth factor ß I and II,
HSA-insulin,
HSA-cachetin,
HSA-epidermal growth factor,
HSA-bursin,
HSA-insulin-like growth factor I,
HSA-insulin-like growth factor II,
HSA-inhibin,
HSA-liver cell growth factor (Gly His Lys),
HSA-nerve growth factor,
HSA-platelet derived growth factor,
HSA-tuftsin and
HSA-interleukins,

the hyphen designating a coupling amide bond between a functional group in HSA

(an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide growth factor in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the HSA molecule carrying one or, preferably, a multitude of the growth factor groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the HSA molecule,

and the corresponding coupled products where the peptide species coupled to the HSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the growth factor.

24. A coupled product according to claim 17, the coupled product being human serum albumin (HSA) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, the coupled product being selected from the group consisting of

HSA-fibronectin cell attachment sequence (Gly Asp Arg)
HSA-fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
HSA-leucine zipper sequence of e.g. c-fos or c-jun,
HSA-helix-turn helix DNA binding sequence,
HSA-protease inhibitor,
HSA-kinase inhibitor,
HSA-muramyl peptide,
HSA-zinc finger sequence,
HSA-B or T cell activating sequence,
HSA-nuclear translocation signal such as Lys Lys Lys Arg Lys,
HSA-membrane translocation signal,
HSA-glycosylation sequence (Asn X Ser/Thr), and
HSA-muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in HSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the

synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the HSA molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the HSA molecule,

and the corresponding coupled products where the coupled peptide is a derivative, fragment or analog thereof.

25. A coupled product according to claim 17, the coupled product being bovine serum albumin (BSA) coupled to a hormone, and in particular being selected from the group consisting of:

BSA-ß-endorphin,
BSA-ß-lipotropin,
BSA-Ø-endorphin,
BSA-gastrin releasing peptide,
BSA-growth hormone releasing factor,
BSA-neurotensin,
BSA-α-melanocyte stimulating hormone,
BSA-adrenocorticotropic hormone,
BSA-vasopressin,
BSA-angiotensin,
BSA-substance P,
BSA-dynorphin,
BSA-bradykinin,
BSA-luteinising hormone releasing hormone,
BSA-parathyroid hormone,
BSA-atrial natriuretic factor,
BSA-atriopeptin,
BSA-calcitonin,
BSA-calcitonin gene related peptide,
BSA-dermorphin,
BSA-kyotorpin,

BSA-auriculin,
BSA-ß-casomorphin,
BSA-tachykinin,
BSA-bombesin,
BSA-caerulein,
BSA-chorionic gonadotropin,
BSA-cholecystokinin,
BSA-casomorphin,
BSA-corticotropin,
BSA-corticotropin releasing factor,
BSA-corticotropin inhibiting peptide,
BSA-somatostatin,
BSA-eukephalin,
BSA-gastric inhibitory peptide,
BSA-gastrin,
BSA-gastrin related peptide,
BSA-thyrotropin releasing hormone,
BSA-glucacon,
BSA-growth hormone,
BSA-growth hormone releasing factor,
BSA-thyroid stimulating hormone,
BSA-prolactin,
BSA-neurokinin A and B,
BSA-neuromedin B, C, K, L, and U,
BSA-neuropeptide Y,
BSA-neurotensin,
BSA-oxytocin,
BSA-pancreastatin,
BSA-vasoactive intestinal peptide,
BSA-secretin,
BSA-galanin,
BSA-pancreatic polypeptide,
BSA-thymosin and
BSA-leucokinin,

the hyphen designating a coupling amide bond between a functional group in BSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the BSA molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the BSA molecule,

and the corresponding coupled products where the peptide species coupled to the BSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone.

26. A coupled product according to claim 17, the coupled product being bovine serum albumin (BSA) coupled to a peptide growth factor and in particular being selected from the group consisting of:

BSA-fibroblast growth factor,
BSA-transforming growth factor α,
BSA-transforming growth factor β I and II,
BSA-insulin,
BSA-cachetin,
BSA-epidermal growth factor,
BSA-bursin,
BSA-insulin-like growth factor I,
BSA-insulin-like growth factor II,
BSA-inhibin,
BSA-liver cell growth factor (Gly His Lys),
BSA-nerve growth factor,
BSA-platelet derived growth factor,
BSA-tuftsin and
BSA-interleukins,

the hyphen designating a coupling amide bond between a functional group in BSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the BSA molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the BSA molecule,

and the corresponding coupled products where the peptide species coupled to the HSA is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the growth factor.

27. A coupled product according to claim 17, the coupled product being bovine serum albumin (BSA) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, and in particular being selected from the group consisting of:

BSA-fibronectin cell attachment sequence (Gly Asp Arg)
BSA-fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
BSA-leucine zipper sequence of e.g. c-fos or c-jun,
BSA-helix-turn helix DNA binding sequence,
BSA-protease inhibitor,
BSA-kinase inhibitor,
BSA-muramyl peptide,
BSA-zinc finger sequence,
BSA-B or T cell activating sequence,
BSA-nuclear translocation signal such as Lys Lys Lys Arg Lys,
BSA-membrane translocation signal,
BSA-glycosylation sequence (Asn X Ser/Thr), and
BSA-muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in BSA (an amino group, a carboxy group, or a hydroxy group converted into a derivative

containing an amino group or a carboxy group), and a functional group of the synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the BSA molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the BSA molecule,

and the corresponding coupled product where the peptide is a derivative, fragment or analogue thereof.

28. A coupled product according to claim 20, the coupled product being purified protein derivative (PPD) coupled to a hormone, and in particular being selected from the group consisting of:

PPD–ß–endorphin,
PPD–ß–lipotropin,
PPD–☺–endorphin,
PPD–gastrin releasing peptide,
PPD–growth hormone releasing factor,
PPD–neurotensin,
PPD–α–melanocyte stimulating hormone,
PPD–adrenocorticotropic hormone,
PPD–vasopressin,
PPD–angiotensin,
PPD–substance P,
PPD–dynorphin,
PPD–bradykinin,
PPD–luteinising hormone releasing hormone,
PPD–parathyroid hormone,
PPD–atrial natriuretic factor,
PPD–atriopeptin,
PPD–calcitonin,
PPD–calcitonin gene related peptide,
PPD–dermorphin,
PPD–kyotorpin,

PPD-auriculin,
PPD-β-casomorphin,
PPD-tachykinin,
PPD-bombesin,
PPD-caerulein,
PPD-chorionic gonadotropin,
PPD-cholecystokinin,
PPD-casomorphin,
PPD-corticotropin,
PPD-corticotropin releasing factor,
PPD-corticotropin inhibiting peptide,
PPD-somatostatin,
PPD-eukephalin,
PPD-gastric inhibitory peptide,
PPD-gastrin,
PPD-gastrin related peptide,
PPD-thyrotropin releasing hormone,
PPD-glucacon,
PPD-growth hormone,
PPD-growth hormone releasing factor,
PPD-thyroid stimulating hormone,
PPD-prolactin,
PPD-neurokinin A and B,
PPD-neuromedin B, C, K, L, and U,
PPD-neuropeptide Y,
PPD-neurotensin,
PPD-oxytocin,
PPD-pancreastatin,
PPD-vasoactive intestinal peptide,
PPD-secretin,
PPD-galanin,
PPD-pancreatic polypeptide,
PPD-thymosin and
PPD-leucokinin,

the hyphen designating a coupling amide bond between a functional group in PPD (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the PPD molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the PPD molecule,

and the corresponding coupled products where the peptide species coupled to the PPD is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone.

29. A coupled product according to claim 20, the coupled product being purified protein derivative (PPD) coupled to a peptide growth factor, and in particular being selected from the group consisting of:

PPD–fibroblast growth factor,
PPD–transforming growth factor α,
PPD–transforming growth factor β I and II,
PPD–insulin,
PPD–cachetin,
PPD–epidermal growth factor,
PPD–bursin,
PPD–insulin–like growth factor I,
PPD–insulin–like growth factor II,
PPD–inhibin,
PPD–liver cell growth factor (Gly His Lys),
PPD–nerve growth factor,
PPD–platelet derived growth factor,
PPD–tuftsin and
PPD–interleukins,

the hyphen designating a coupling amide bond between a functional group in PPD (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the PPD molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the PPD molecule,

and the corresponding coupled products where the peptide species coupled to the PPD is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the growth factor.

30. A coupled product according to claim 20, the coupled product being purified protein derivative (PPD) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, and in particular being selected from the group consisting of:

PPD–fibronectin cell attachment sequence (Gly Asp Arg)
PPD–fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
PPD–leucine zipper sequence of e.g. c–fos or c–jun,
PPD–helix–turn helix DNA binding sequence,
PPD–protease inhibitor,
PPD–kinase inhibitor,
PPD–muramyl peptide,
PPD–zinc finger sequence,
PPD–B or T cell activating sequence,
PPD–nuclear translocation signal such as Lys Lys Lys Arg Lys,
PPD–membrane translocation signal,
PPD–glycosylation sequence (Asn X Ser/Thr), and
PPD–muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in PPD (an amino group, a carboxy group, or a hydroxy group converted into a derivative

containing an amino group or a carboxy group) and a functional group of the synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the PPD molecule carrying one or, preferably, a multitude of the groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the PPD molecule,

and the corresponding coupled products where the peptide is a derivative, fragment, or analog thereof.

31. A coupled product according to claim 21, the coupled product being tetanus toxoid (TNT) coupled to a hormone, and in particular being selected from the group consisting of:

TNT-β-endorphin,
TNT-β-lipotropin,
TNT-Θ-endorphin,
TNT-gastrin releasing peptide,
TNT-growth hormone releasing factor,
TNT-neurotensin,
TNT-α-melanocyte stimulating hormone,
TNT-adrenocorticotropic hormone,
TNT-vasopressin,
TNT-angiotensin,
TNT-substance P,
TNT-dynorphin,
TNT-bradykinin,
TNT-luteinising hormone releasing hormone,
TNT-parathyroid hormone,
TNT-atrial natriuretic factor,
TNT-atriopeptin,
TNT-calcitonin,
TNT-calcitonin gene related peptide,
TNT-dermorphin,

TNT-kyotorpin,
TNT-auriculin,
TNT-β-casomorphin,
TNT-tachykinin,
TNT-bombesin,
TNT-caerulein,
TNT-chorionic gonadotropin,
TNT-cholecystokinin,
TNT-casomorphin,
TNT-corticotropin,
TNT-corticotropin releasing factor,
TNT-corticotropin inhibiting peptide,
TNT-somatostatin,
TNT-eukephalin,
TNT-gastric inhibitory peptide,
TNT-gastrin,
TNT-gastrin related peptide,
TNT-thyrotropin releasing hormone,
TNT-glucacon,
TNT-growth hormone,
TNT-growth hormone releasing factor,
TNT-thyroid stimulating hormone,
TNT-prolactin,
TNT-neurokinin A and B,
TNT-neuromedin B, C, K, L, and U,
TNT-neuropeptide Y,
TNT-neurotensin,
TNT-oxytocin,
TNT-pancreastatin,
TNT-vasoactive intestinal peptide,
TNT-secretin,
TNT-galanin,
TNT-pancreatic polypeptide,
TNT-thymosin and

TNT-leucokinin,

the hyphen designating a coupling amide bond between a functional group in TNT (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the TNT molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the TNT molecule,

and and the corresponding coupled products where the peptide species coupled to the TNT is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the hormone.

32. A coupled product according to claim 21, the coupled product being tetanus toxoid (TNT) coupled to a peptide growth factor, and in particular being selected from the group consisting of:

TNT-fibroblast growth factor,
TNT-transforming growth factor α,
TNT-transforming growth factor β I and II,
TNT-insulin,
TNT-cachetin,
TNT-epidermal growth factor,
TNT-bursin,
TNT-insulin-like growth factor I,
TNT-insulin-like growth factor II,
TNT-inhibin,
TNT-liver cell growth factor (Gly His Lys),
TNT-nerve growth factor,
TNT-platelet derived growth factor,
TNT-tuftsin and
TNT-interleukins,

the hyphen designating a coupling amide bond between a functional group in TNT (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group) and a functional group of the peptide hormone in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the TNT molecule carrying one or, preferably, a multitude of the hormone groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the TNT molecule,

and the corresponding coupled products where the peptide species coupled to the TNT is a precursor, a fragment, a derivative or an analogue (e.g. an antagonist or an agonist) of the growth factor.

33. A coupled product according to claim 21, the coupled product being tetanus toxoid (TNT) coupled to a peptide which is a synthetic fragment of a protein with defined biological effect, and in particular being selected from the group consisting of:

TNT-fibronectin cell attachment sequence (Gly Asp Arg)
TNT-fibronectin cell attachment sequence (Ser Gly Asp Arg Gly),
TNT-leucine zipper sequence of e.g. c-fos or c-jun,
TNT-helix-turn helix DNA binding sequence,
TNT-protease inhibitor,
TNT-kinase inhibitor,
TNT-muramyl peptide,
TNT-zinc finger sequence,
TNT-B or T cell activating sequence,
TNT-nuclear translocation signal such as Lys Lys Lys Arg Lys,
TNT-membrane translocation signal,
TNT-glycosylation sequence (Asn X Ser/Thr), and
TNT-muramyl peptide,

the hyphen designating a coupling amide bond between a functional group in TNT (an amino group, a carboxy group, or a hydroxy group converted into a derivative

containing an amino group or a carboxy group) and a functional group of the synthetic fragment in question (an amino group, a carboxy group, or a hydroxy group converted into a derivative containing an amino group or a carboxy group), the TNT molecule carrying one or, preferably, a multitude of the synthetic fragment groups coupled thereto, the multitude being a number less than or equal to the number of the relevant functional groups on the TNT molecule,

and the corresponding coupled products where the peptide is a derivative, fragment or analogue thereof.

34. A method of producing an antibody specific for a peptide such as a peptide hormone, a peptide growth factor, or a peptide with another defined biological effect, which metod comprises immunizing an animal with a coupled product according to any of the claims 8–33, in which the molecule coupled to the proteinaceous substance is the peptide to which the specific antibody is to be raised, or an immunogenically effective part of the peptide, to make cells in the animal produce an antibody specific for said peptide, and obtaining such cells from the animal, or isolating the antibody from serum or plasma of the animal.

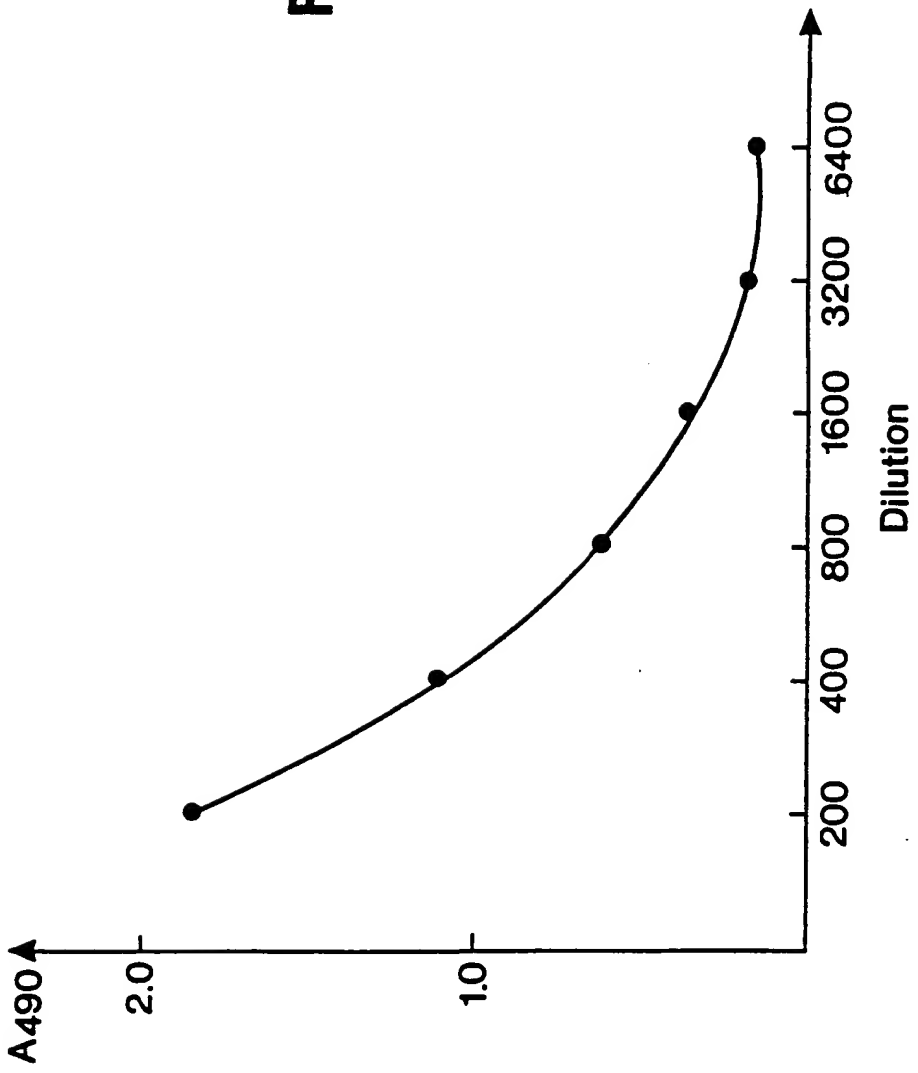
35. A method of purifying antibodies against a peptide, comprising applying a medium containing the antibodies to a suspension or solution of a coupled product according to any of the claims 8–33 carrying a coupled peptide, to bind the peptide–specific antibodies to the peptide moiety of the coupled product, washing to remove unbound antibodies, cleaving the antibodies from the peptide moiety, and separating the antibodies from the medium, e.g. by dialysis.

36. The use of a coupled product according to any of the claims 8–33 as a vaccine.

37. The use of a coupled product according to any of the claims 8–33 as a matrix material or a carrier molecule.

38. Products, including peptides and peptide derivatives, prepared by the method according to any of the claims 1–7.

Fig. 1



INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00311

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 1/04, 15/00, A 61 K 39/385

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A1, 0329994 (BOEHRINGER MANNHEIM GMBH) 30 August 1989, see the whole document --	1-38
X	EP, A2, 0175613 (MERCK & CO.INC.) 26 March 1986, see the whole document --	1-38
X	Laboratory techniques in biochemistry and molecular biology, vol. 19, 1988, R.H. Burdon et al. Eds., ELSEVIER, Amsterdam New York Oxford, pages 95-144 --	1-38
X	EP, A1, 0284492 (RHONE MERIEUX) 28 September 1988, see the whole document --	1-38

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th March 1991	1991 -03- 14
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Elisabeth Carlborg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECCND SHEET)

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A	EP, A2, 0243929 (F. HOFFMANN-LA ROCHE & CO.) 4 November 1987, see the whole document --	1-33
A	US, A, 3772264 (ERNST BAYER ET AL) 13 November 1973, see the whole document --	1-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 90/00311

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 91-01-31
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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